LNP encapsulation efficiency, size & more from a single read on Stunner AF

Introduction

Encapsulation efficiency (EE%) is a critical step in lipid nanoparticle (LNP) characterization, but old-school fluorescent dye-based assays are slow, tedious, and low-throughput - putting a hitch in development. And when you add on sizing information gathered from samples one-by-one, development can slow to a crawl.

By combining fluorescence detection with UV/Vis, and rotating angle dynamic light scattering (RADLS) in a single platform, Stunner AF delivers EE%, total RNA, free RNA, size, polydispersity, and particle concentration – all in a low-volume, high-throughput plate-based platform (Figure 1).

This app note details how Stunner AF simplifies LNP characterization, saves you time, and delivers high precision results in a single read, without surfactants or disruption.

Stunner AF brings it all together

Stunner AF is the only system that pulls together fluorescence, UV/Vis concentration and dynamic light scattering on the same tiny 2 µL wells (Figure 2). Fluorescence is designed to work with nucleic acid sensing dyes in order to quantify free RNA present in a sample. Total RNA is detected by measuring UV/Vis absorbance and then using advanced Unmix algorithms to deconvolute RNA absorbance from other components, like cholesterol or turbidity. Stunner AF is kitted out with RADLS to gather static and dynamic light scattering data from multiple angles in each read. In dynamic light scattering multiple angles are useful for detecting the size and PDI of larger particles (like LNPs) and being highly sensitive to larger aggregates floating around. Gathering multiple angles of data for static light scattering is a MALS-based approach to give Stunner AF the info it needs to calculate particle concentration for your LNPs.

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

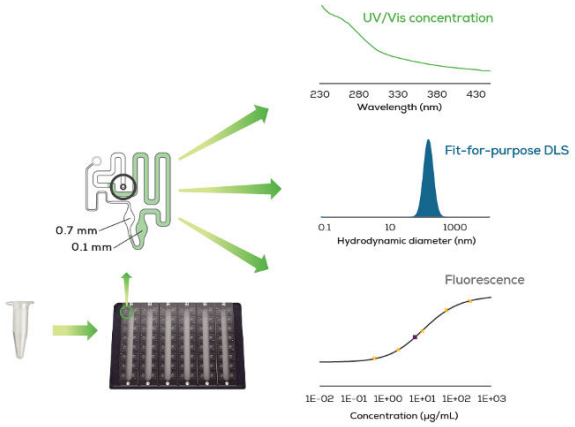

Figure 2: Microfluidic circuits in a 96-well, plate-based consumable allow Stunner AF to gather the info it needs for EE%, size, PDI, free and total RNA quantification.

One single measurement

Twelve LNP samples using a variety of cationic lipids were run on the RNA-LNP EE application to simultaneously characterize encapsulation efficiency by free and total RNA quantification, sizing, PDI, and particle concentration (Figure 3). For 12 samples the total prep time for making a 6-point standard curve, diluting samples and loading the plate was 26 minutes, and reading the plate took 1 hour 15 minutes of hands-off time.

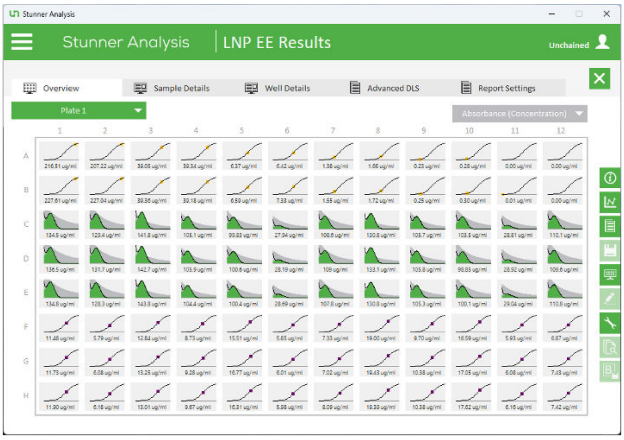

Figure 3: Results for 12 LNP samples. The first two rows are a reference standard curve in quadruplicates. Rows C, D, E are triplicate wells measuring total RNA concentration by UV/Vis. Rows F, G, H are triplicate wells measuring free RNA concentration by fluorescence.

Free RNA was quantified by RiboGreen® against a 6-point standard curve (see Methods section). For every well, green fluorescence was measured and automatically evaluated against a 4-parameter fit of the reference standard samples. The median %CV across the twelve triplicate samples was 2.7%.

Total RNA concentration was determined by UV/Vis without the use of any surfactants or particle disruption. The median %CV for payload concentration (total RNA – free RNA) was 0.6% (Figure 4).

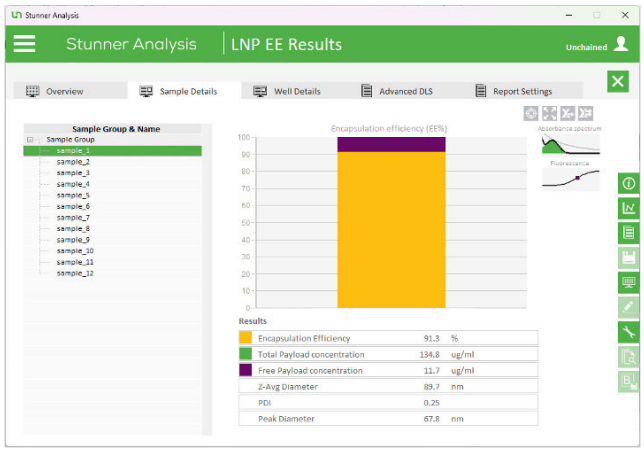

Figure 4: A characterization snapshot for each LNP sample summarizes EE%, total and free payload, concentration and sizing results in a single view.

From the quantification done by fluorescence and UV/Vis, it's easy for Stunner AF to do the math for you and calculate encapsulation efficiency on every sample (Figure 5). Stunner AF uses the median concentration value determined for free and total RNA to calculate EE%. Since each sample is analyzed by multiple biophysical techniques, Stunner AF summarizes everything to show EE% next to quantification and sizing data, all at once. A deep dive into the details of each well is a single click away or available in a configurable Excel report.

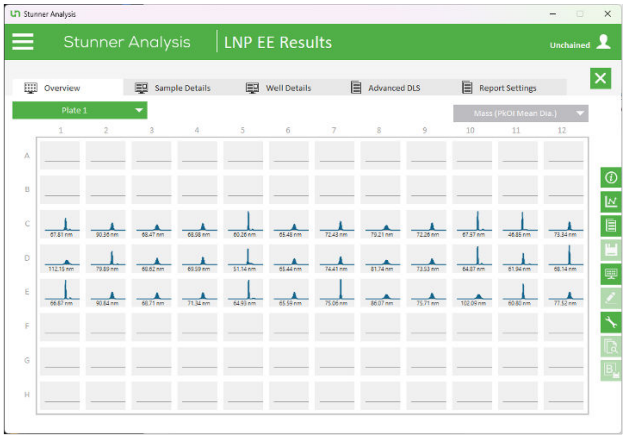

Figure 5: Mass distribution of sizes for the twelve LNP samples are determined from the undiluted, dye-free replicates.

Stunner AF gives you the most sensitive DLS on the market, utilizing cutting edge rotating angle dynamic light scattering (RADLS). A full RADLS analysis on an LNP uses 7 angles that pick up back- and forward-scattering light, giving you an angle-independent analysis on your sample size, along with particle count. To screen particle size fast, Stunner AF can still keep things simple and power through sizing data from a single angle.

How Stunner AF stacks up

Much like Stunner AF, the classic method for reading EE% also relies upon using a fluorescent dye for free RNA quantification but requires a surfactant to disrupt particles to measure total RNA concentration. Stunner AF was benchmarked against the classic EE% assay performed on a fluorescent plate reader for three LNP formulations: one containing SM-102, one with MC3, and a cationic LNP. In addition, the SM-102 formulation was also challenged with low and high spike-in amount of free RNA which were directly reflected in the EE% measurements. EE% values on both systems (Figure 6) have comparable results.

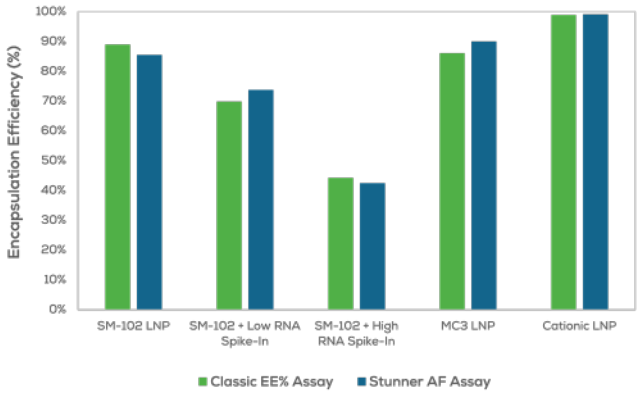

Figure 6: Side-by-side comparison of the classic EE% assay done on a fluorescent plate reader and when measured by Stunner AF for three LNP formulations plus two free RNA spike-in conditions.

Beyond matching up to 1-at-a-time EE% methods, dramatically less hands-on time and total time is required for characterization on Stunner AF (Tables 1 and 2) show the time savings for 12 samples. Most hands-on time savings comes from teaming up concentration measurements with plate-based RADLS, where Stunner AF can reduce hands-on time commitments by hours. Thanks to the super-simple design of the Stunner AF EE% assay and the chance to skip the addition of surfactants and an incubation step, even the hands-on time required for the EE% assay on Stunner AF is less – about an hour is saved by the Stunner AF method.

Classic Method	Hands-on Time	Total Time
One-by-One DLS	1-2 hours	>2 hours
Full Dye-Based EE% Assay	1.5 hours	1.5 hours
Total	> 2 hours	~3 hours

Table 1: Estimated time to test 12 samples in triplicate for EE%, size, payload concentration and particle concentration.

Stunner AF	Hands-on Time	Total Time
EE% & Size	30 minutes	1.5 hours

Table 2: Time to test 12 samples in triplicate for EE%, size, payload concentration and particle concentration.

Painting the full picture of LNP production

To show Stunner AF in action, three different LNP formulations were characterized using the RNA-LNP EE application (Figure 7). Two ionizable (SM-102 and ALC-0315) lipid formulations showed particles with a Z-average diameter of approximately 67 nm, while the cationic (DDAB) LNP formulation produced larger particles with a diameter of 106 nm. All three formulations gave encapsulation efficiencies above 90%. However, for this preparation of LNPs the payload concentration of RNA was significantly lower for DDAB as it only encapsulated about 13 µg/mL of RNA, which is about a third of the average 41 µg/mL of RNA encapsulated by both ionizable LNP formulations. Particle concentrations had an inverse relationship to average diameter, as the smaller ionizable LNPs had higher concentrations of particles and vice versa.

Each of these three formulations was also used to compare EE% performance between a fluorescence plate reader and Stunner AF. Across the three formulations encapsulation efficiency was within 4 percentage points and both methods agreed with an encapsulation efficiency greater than 90%.

Conclusions

Stunner AF is an unparalleled tool for LNP analysis, with RADLS to tease out the presence of previously invisible aggregates, and UV-Vis + fluorescence to give you encapsulation efficiency and payload information. Time and effort can be saved by using Stunner AF to combine sizing and RNA quantification in a single instrument designed to replace rate-limiting assays in the LNP characterization workflow. Complete characterization with no surfactants, simple setup and rapid data generation make high-throughput screening a reality, and with more information than ever before.

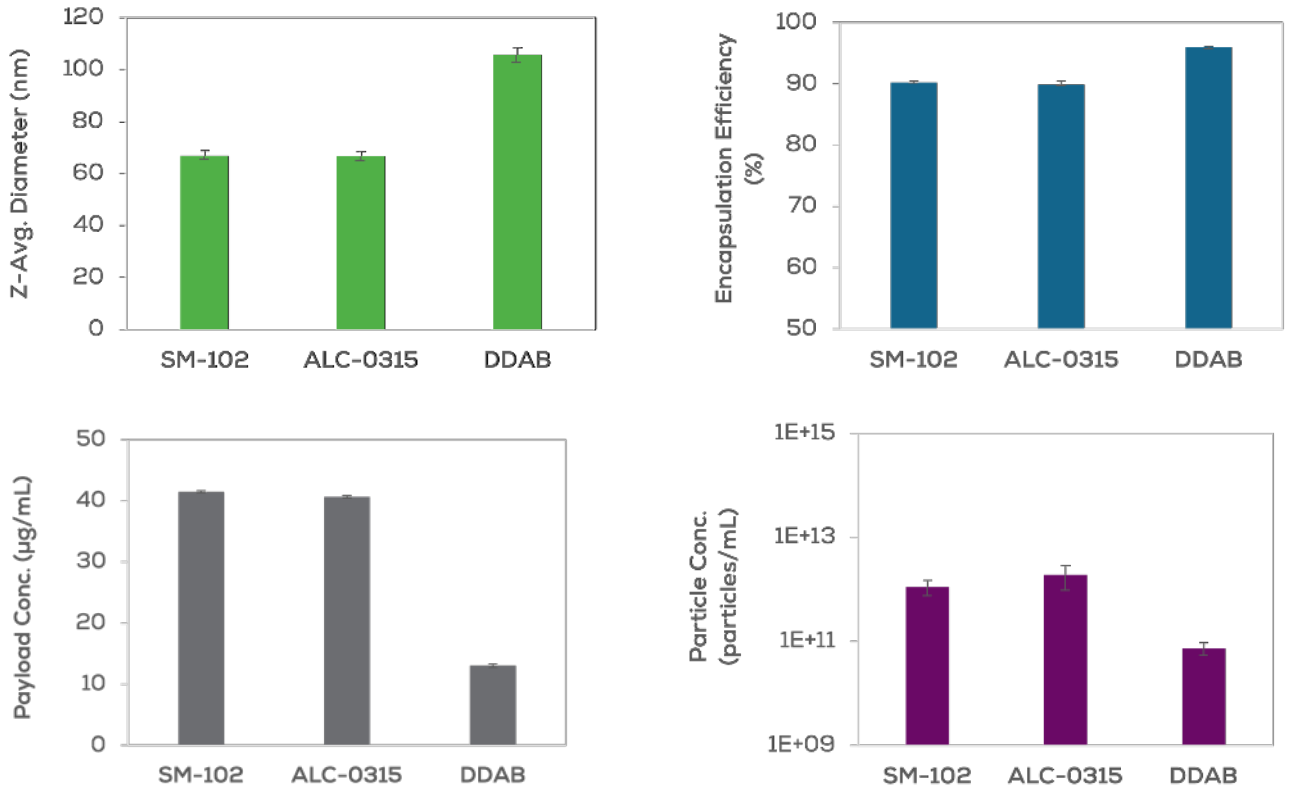

Figure 7: Comparison of three LNP formulations across size, EE%, payload concentration, and particle concentration.

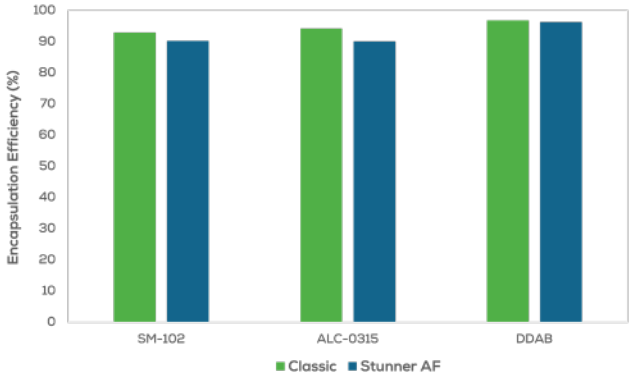

Figure 8: Encapsulation efficiency for the three LNP formulations as measured by the classic EE% assay on a fluorescent plate reader or measured by Stunner AF.

Methods

The data in this application note was generated with the RNA-LNP EE or RNA-LNP EE Screen applications available in the Nanoparticles menu for Stunner AF.

Sample layout varied for each experiment. As an example of a plate layout, the plate shown in (Figure 9) has triplicate UV/Vis samples (green), triplicate fluorescent samples (purple) and quadruplicate reference samples (yellow) for a standard curve with 5 points and a zero point. Auto-blanking was used where blanks for UV/Vis (blue) were omitted. If desired, single replicates can be used for all samples with single zero and non-zero fluorescence samples. If even higher throughput is desired (e.g. >44 samples per experiment), reference samples for standard curves, fluorescence samples, UV/Vis samples, and blanks can be located across multiple plates within a single experiment.

RNA payloads used were a 5'-methoxypseudou-ridine eGFP mRNA with concentration factor = 41.65 (ng/µL)*cm or polyA with concentration factor = 29.4 (ng/µL)*cm or the RNA aptamer Broccoli with concentration factor = 40 ng/µL)*cm. Stunner AF automatically makes adjustments for hypochromicity for the impact of encapsulation on RNA when quantified by UV/Vis.

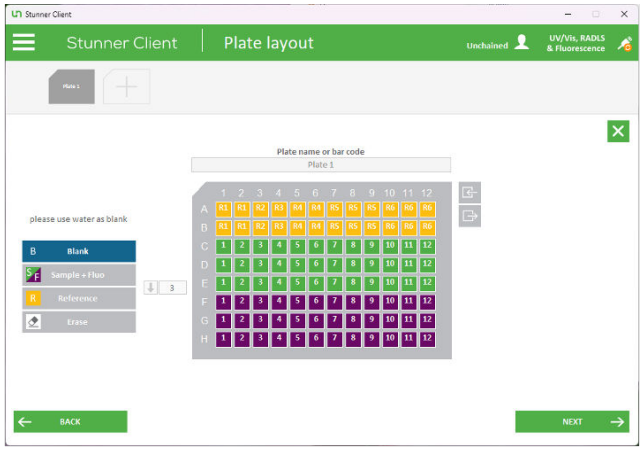

Figure 9: Stunner AF plate layout exhibiting triplicate UV/Vis samples (green), triplicate fluorescent samples (purple) and quadruplicate reference samples (yellow).

For 6-point standard curves, a High Range or Mid Range dilution series was typically used:

Unchained Master Mix was combined with RiboGreen® at a 1:60 dilution to create Working Solution. For 1 plate containing 12 LNP samples, you need at least 360 µL of Working Solution. To prepare an excess of 390 µL of Working Solution: add 6.5 µL of RiboGreen® to 383.5 µL of Master Mix.

5 µL of each fluorescent sample (purple in plate maps) or reference standards (yellow) was added to 20 µL of combined Working Solution to prepare samples for fluorescence quantification. Neither master mix nor dye was added to the UV/Vis samples (green) as they are measured for total RNA payload and sizing at stock, undiluted concentrations.

LNPs were prepared on Sunscreen using a variety of formulations incorporating SM-102, ALC-0315, DLin-MC3-DMA, and cationic lipids alongside PEGylated lipids, cholesterol, and helper lipids.

	High Range	Mid Range	Low Range
Dilution Factor (1/x)	5	4	3
Standard 1	250 µg/mL	100 µg/mL	20 µg/mL
Standard 2	50 µg/mL	25 µg/mL	6.67 µg/mL
Standard 3	10 µg/mL	6.25 µg/mL	2.22 µg/mL
Standard 4	2 µg/mL	1.56 µg/mL	0.74 µg/mL
Standard 5	0.4 µg/mL	0.39 µg/mL	0.25 µg/mL
Standard 6	0 µg/mL	0 µg/mL	0 µg/mL

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