

# Hassle-free nanoparticle characterization with Stunner

## Introduction

Characterization of nanoparticles and lipid nanoparticles (LNPs) covers many parameters but two of the most important are payload concentration and size. Quantifying payload concentration is key to knowing how much drug is being delivered, and nanoparticles must be the right size to be effective in getting their payload where they need to go.

Different assays are used to quantify RNA, DNA, protein, or small molecule payloads, but most rely on complicated disruption workflows, costly dyes, and wasteful standard curves. On top of the extra work of disrupting nanoparticles, the detergents used can interfere with a dye's fluorescent or colorimetric signal. UV/Vis absorbance has been around forever for RNA, DNA, protein and small molecule quantification in purified samples, but the high turbidity of nanoparticles makes samples too cloudy for most UV/Vis spectrophotometers and keeps them from getting accurate readings.

Dynamic light scattering (DLS) is a common analytical technique for the sizing of nanoparticles, but many DLS instruments require tons of sample and hefty hands-on time. One-trick DLS instruments also don't give any information on payload or can't say much about particle concentrations. Dedicating tons of time to characterize just a few aspects of a nanoparticle slows down the development of life-saving gene therapies, vaccines and biopharmaceuticals.

Stunner combines high-throughput, short pathlength UV/Vis and advanced Unmix analysis with DLS to quantify payloads and determine size of any nanoparticle in a single step (Figure 1).

For LNP samples, Stunner's built-in applications for RNA-LNP, DNA-LNP, or protein-LNP payloads makes sizing and quant simple and easy. Stunner cuts through the turbidity of an LNP with Unmix analysis to separate out the absorbance of each pre-set payload from any particle or buffer absorbance.

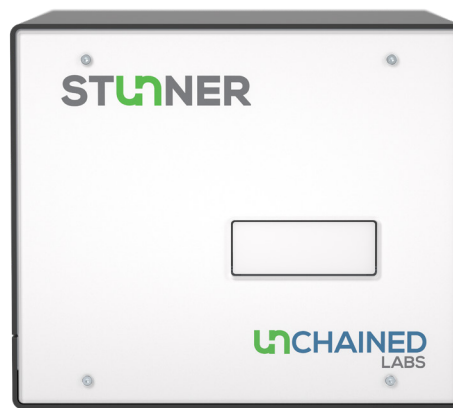


Figure 1: Stunner: the one-and-only combination of UV/Vis and dynamic light scattering (DLS) to characterize any nanoparticle.

To go beyond LNPs, the Custom Nanoparticle application lets you show Stunner exactly what to look for and how to quantify it. Teach Stunner the spectra for the analytes that make up your custom particle and payload and you've got a crazy simple shortcut to quantification.

Stunner gives researchers a non-destructive way to quant any payload and determine the size of LNPs or other nanoparticles in about a minute using only 2  $\mu$ L of sample. It reads both UV/Vis and DLS on up to 96 samples of LNPs in just 1 hour. When even higher throughput is needed, Stunner is automation-friendly with its SBS format plates. For regulatory environments, Stunner's UV/Vis performance can be verified for US and European Pharmacopeia compliance and can be souped-up with 21 CFR Part 11 tools.

This app note describes how Stunner quantifies payload, particle size, size distribution and lipid absorbance of RNA-, DNA-, protein- and drug-loaded LNPs.

## Methods

1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 2-Oleoyl-1-palmi-

toyl-sn-glycero-3-phosphoethanolamine (POPE), cholesterol (Chol), and 1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG) lipid stocks were made in ethanol. Calf liver RNA and calf thymus DNA were filtered with a 10 kD MWCO filter and the retentate used for LNP production.

RNA-, DNA-, and lysozyme-loaded LNPs were made using the NanoAssemblr® Ignite by Precision NanoSystems Inc. (Vancouver, BC, Canada). All LNPs were composed of DOTAP:POPE:Chol:DMG-PEG dissolved in ethanol at 50:10:38.5:1.5 molar ratio and made with a total flow rate (TFR) of 12 mL/min and an aqueous-to-organic flow rate ratio (FRR) of 3:1. RNA- and DNA-LNPs were made with nitrogen-to-phosphate ratio (N/P) of 5, 10, and 50 by adding 100, 50, and 10 µg/mL, respectively, nucleic acid to the aqueous phase (100 mM citrate buffer, pH 4) and a final lipid concentration of 4 mM. Lysozyme-LNPs were made with an aqueous phase of phosphate-buffered saline (PBS) with initial lysozyme concentrations as shown and a final lipid concentration of 5 mM. After production, the LNPs were buffer-exchanged into PBS, pH 7.4 to reduce residual organic solvent.

Doxorubicin-loaded PEGylated and empty control liposomes from Avanti Polar Lipids (Alabaster, AL, USA) were diluted into a 2-fold dilution series. The spectra were measured using Stunner's Stored Spectrum feature and used to create an analyte for the Custom Nanoparticle app.

Ultra Uniform PEG-carboxyl gold nanosphere standards from nanoComposix (San Diego, CA, USA) were used to validate Stunner's DLS system.

Payload quantification, lipid & buffer absorbance, hydrodynamic size and polydispersity were assessed using the appropriate Stunner LNP application. All Stunner measurements were performed with 4 replicates and 2 µL of sample with PBS as a blank. A buffer viscosity and refractive index of 1.002 cP and 1.334, respectively, at 20°C and 4 DLS acquisitions of 5 seconds each were used for DLS.

## Results

UV/Vis absorbance spectra of LNPs are dominated by light scattering across the entire ultraviolet and visible range, making it difficult or impossible for

most UV/Vis spectrometers to quantify payloads of these challenging samples. This high level of light scattering can be seen by eye as turbidity or cloudiness. Stunner's suite of LNP apps use Unmix algorithms to deconvolute the absorbance spectra of LNPs to determine the individual contributions of turbidity, payload, lipid and other components to the total signal. The built-in apps are each designed to detect specific payload molecules: RNA, DNA or protein. The Custom Nanoparticle app allows the user to piece together their nanoparticle from the absorbance of each component, including the payload, so it can be deconvoluted and quantified from the overall absorbance spectra. All the deconvolution done by Unmix is based on the absorbance spectra of the components, so it can be done without using empty nanoparticles as a blank. A water control is the only thing Stunner needs for nanoparticle quant.

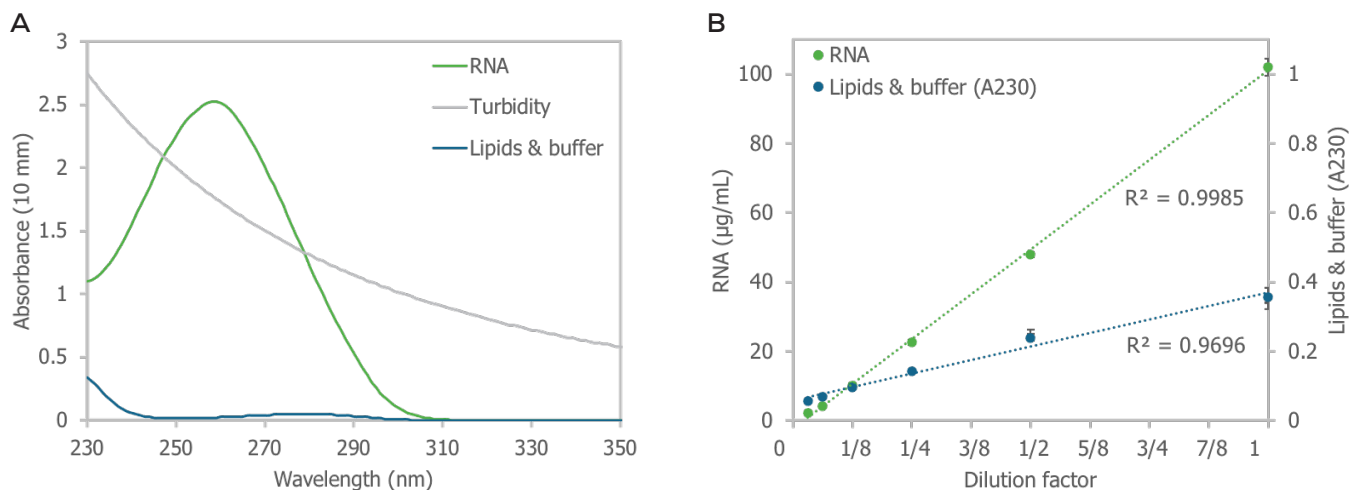
Lipid recovery assays typically rely on complicated liquid chromatography (LC) methods.<sup>1</sup> Since components of LNPs can absorb ultraviolet light, they can be quantified and deconvoluted as part of Stunner's nanoparticle applications. These absorbance values could supplement traditional LC techniques, especially to speed up lipid recovery screens of large numbers of samples.

Verifying particle size with DLS is a key analytical technique for all nanoparticles, especially for formulation and stability studies.<sup>2</sup> Other DLS instruments require significant volumes of sample or can only measure one sample at a time, making it difficult to screen large numbers of formulations or LNPs. Stunner performs UV/Vis and DLS measurements on up to 96 samples in less than 1 hour, using only 2 µL per sample.

### RNA-LNP

Stunner's RNA-LNP app breaks down the particle's absorbance signal and quantifies the total RNA concentration in the sample while also reporting the absorbance contribution from turbidity and a combined lipid & buffer component (Figure 2A).

Stunner's quantification of RNA-LNPs is extremely reproducible. A 2-fold dilution series of RNA-loaded LNPs showed a high degree of linearity and precision for RNA concentration with  $R^2 > 0.99$  and CVs

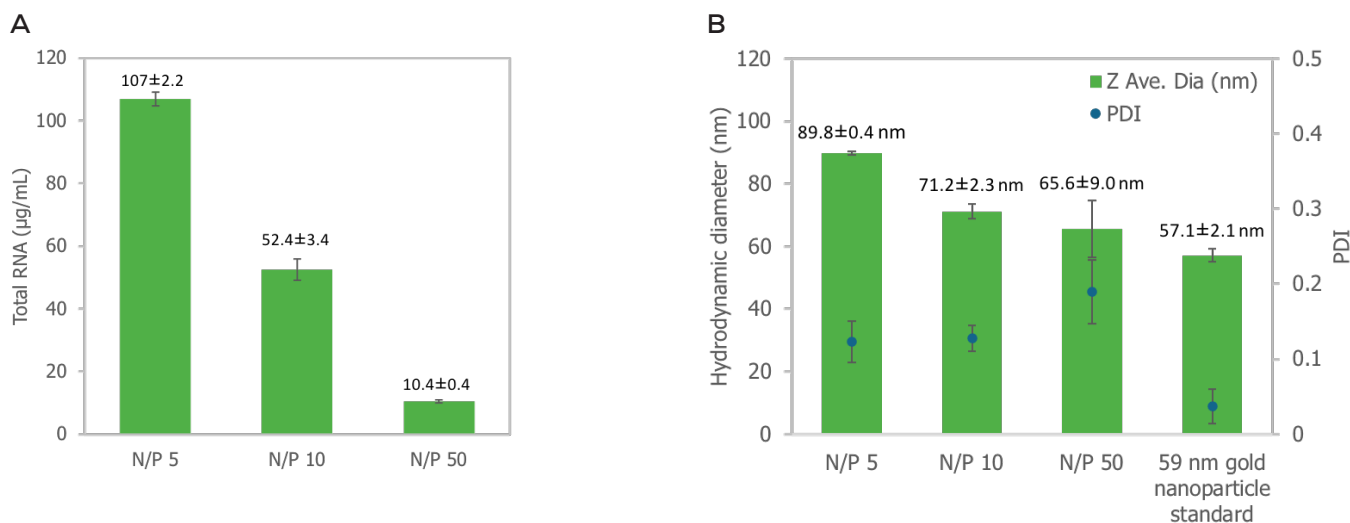


**Figure 2:** The RNA-LNP application deconvolutes the nanoparticle's UV/Vis absorbance spectrum into its components: RNA, turbidity and lipids & buffer (**A**). Stunner's RNA quantification and lipid & buffer absorbance measurements were linear for a dilution series of RNA-loaded LNPs with N/P = 5 (**B**). Error bars are  $\pm 1$  standard deviation (SD).

typically <5% (**Figure 2B**). The absorbance from lipids & buffer at 230 nm was also highly linear, relative to dilution factor, with  $R^2 > 0.96$ . By quantifying RNA-LNPs with UV/Vis absorbance and deconvolution, without detergents or dyes, Stunner can non-destructively quantify total RNA and lipid absorbance, typically proportional to lipid concentration.

RNA quant and particle size are both critical attributes for the assessment of RNA-loaded LNPs.<sup>3</sup> Stunner's combination of UV/Vis and DLS determines both attributes on the same samples during an experiment. RNA-LNPs with different N/P ratios but the same microfluidic parameters and lipid formulations had different RNA concentrations (**Figure 3A**). The

N/P=10 sample contained half and the N/P=50 sample contained about 1/10<sup>th</sup> the RNA of the N/P=5 sample. The size of the 3 RNA-LNPs varied similarly to their payload concentration (**Figure 3B**). The N/P=5 sample had the largest average hydrodynamic diameter and the N/P=50 sample had the smallest. The PDI for the N/P=50 sample, 0.19, was higher than that of the N/P=5 sample, 0.12, indicating the smaller, lower RNA concentration sample was also more polydisperse. The N/P=10 sample size was between the other samples and the polydispersity was similar to the N/P=5 sample. To validate DLS size for the RNA-LNPs, a 59 nm hydrodynamic diameter gold nanoparticle standard had an average hydrodynamic diameter of  $57 \pm 2.1$  nm by Stunner's DLS and an average PDI of 0.04.



**Figure 3:** Stunner's RNA quantifications (**A**) and size and polydispersity of (**B**) RNA-loaded LNPs were highly reproducible for varying N/P ratios. Error bars are  $\pm 1$  standard deviation (SD).

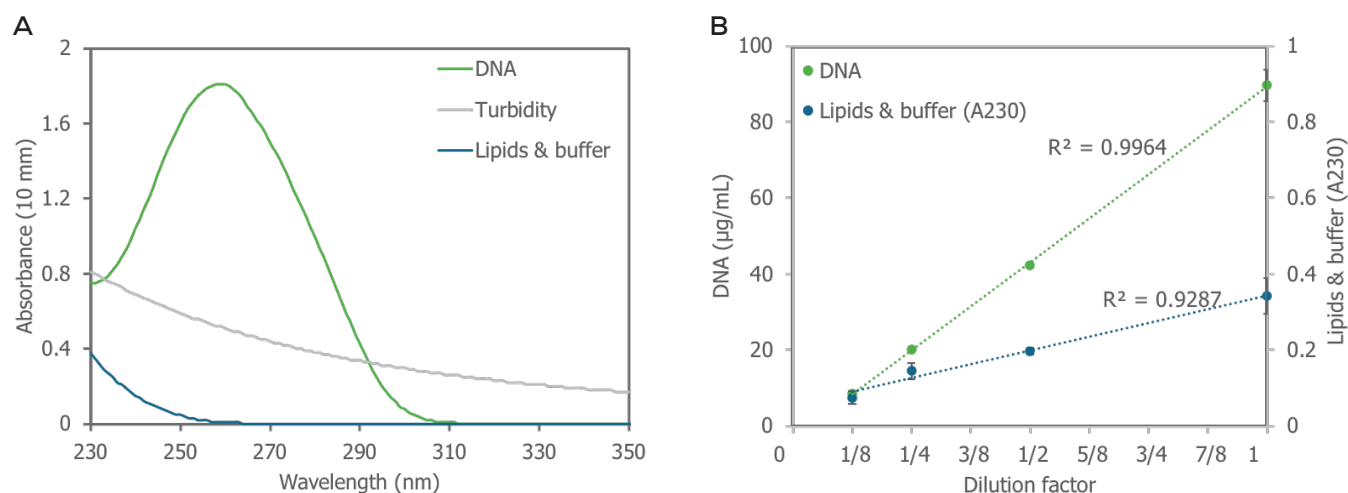


Figure 4: The DNA-LNP application deconvolutes the nanoparticle's UV/Vis absorbance spectrum into its components: DNA, turbidity and lipids & buffer (A). Stunner's DNA quantification and lipid & buffer absorbance measurements were linear for a dilution series of DNA-loaded LNPs with N/P=5 (B). Error bars are  $\pm 1$  standard deviation (SD).

## DNA-LNP

Stunner's DNA-LNP app deconvolutes the sample's absorbance spectrum and quantifies the DNA in the sample. It also reports the absorbance due to lipids & buffer and the turbidity contribution (Figure 4A). A 2-fold dilution series of DNA-loaded LNPs with N/P=5 showed good linearity and precision for DNA quants with  $R^2 > 0.99$  and CVs  $< 5\%$  (Figure 4B). The absorbance from lipids & buffer at 230 nm was also linear with  $R^2 > 0.92$ .

DNA-LNPs with different N/P ratios but the same microfluidic parameters and lipid formulations had different DNA quants (Figure 5A). The N/P=10 sample contained approximately half and the N/P=50 sample contained about 1/10<sup>th</sup> the RNA

of the N/P=5 sample. The size of the 3 RNA-LNPs varied as well (Figure 5B). Even though the N/P=5 sample had the smallest average hydrodynamic diameter and the N/P=50 sample had the largest, the SDs of all 3 samples were too large to determine if the differences were statistically significant. The PDI of the N/P=50 sample was  $> 0.2$ , indicating that this sample was highly polydispersed. Monodispersed samples tend to have PDIs  $< 0.1$ .

## Protein-LNP

Producers of protein-loaded liposomes or LNPs typically use colorimetric dyes or HPLC, combined with particle disruption, to quantify payloads.<sup>4</sup> Both of these methods have their own disadvantages. HPLC is time-consuming and uses a large volume

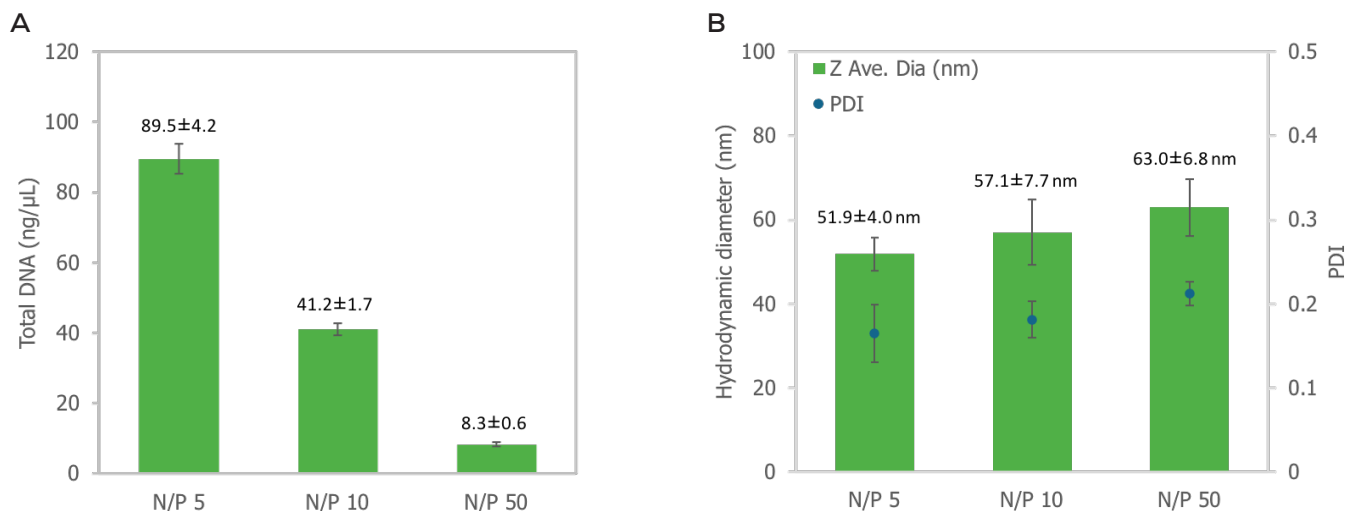


Figure 5: Stunner's DNA quantifications (A) and size and polydispersity (B) of DNA-loaded LNPs were reproducible for varying N/P ratios. Error bars are  $\pm 1$  standard deviation (SD).

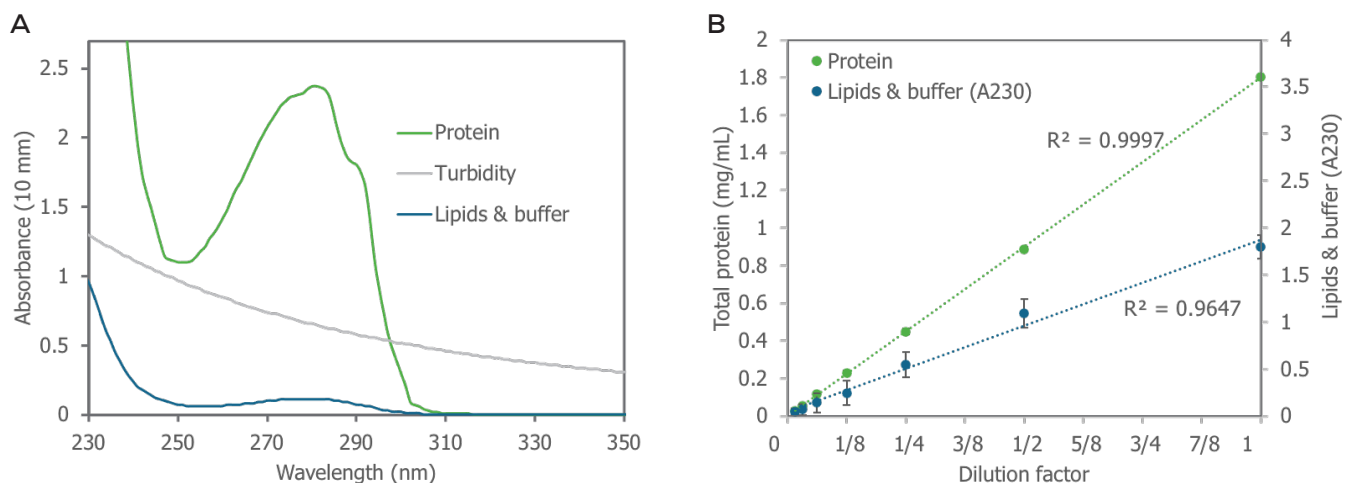


Figure 6: The Protein-LNP application deconvolutes the nanoparticle’s UV/Vis absorbance spectrum into its components: protein, turbidity and lipids & buffer (A). Stunner’s protein quantification and lipid & buffer absorbance measurements were linear for a dilution series of protein-loaded LNPs with initial lysozyme concentrations of 3.5 mg/mL (B). Error bars are  $\pm 1$  standard deviation (SD).

of sample. Colorimetric assays are prone to interference by lipids and detergents and need empty nanoparticles as part of the controls and standards. Stunner needs no detergents, no standard curves, and no empty controls for nanoparticle quantification and characterization.

Stunner’s Protein-LNP app uses the UV/Vis absorbance spectrum of a protein, which has an absorbance peak at 280 nm, to deconvolute the overall UV/Vis absorbance of a protein-loaded LNP into the contribution from the protein, turbidity and other components (Figure 6A). Based on the deconvoluted spectra and the protein’s E1%, or extinction coefficient, Stunner quantifies the overall protein payload concentration in the LNP.

It also reports the absorbance due to particle and buffer components.

A 2-fold dilution series of lysozyme-loaded LNPs with initial protein concentration of 3.5 mg/mL showed good linearity and precision for protein quants with  $R^2 > 0.99$  and CVs  $< 5\%$  (Figure 6B). The absorbance from lipids & buffer at 230 nm was also linear with  $R^2 > 0.96$ .

Lysozyme-loaded LNPs with initial concentrations but the same microfluidic parameters and lipid formulations had protein quants in proportion to their initial concentration (Figure 7A). Compared to the 35 mg/mL sample, the 17.5 mg/mL sample contained approximately half the protein and the 3.5 mg/mL sample contained about 1/10<sup>th</sup> the

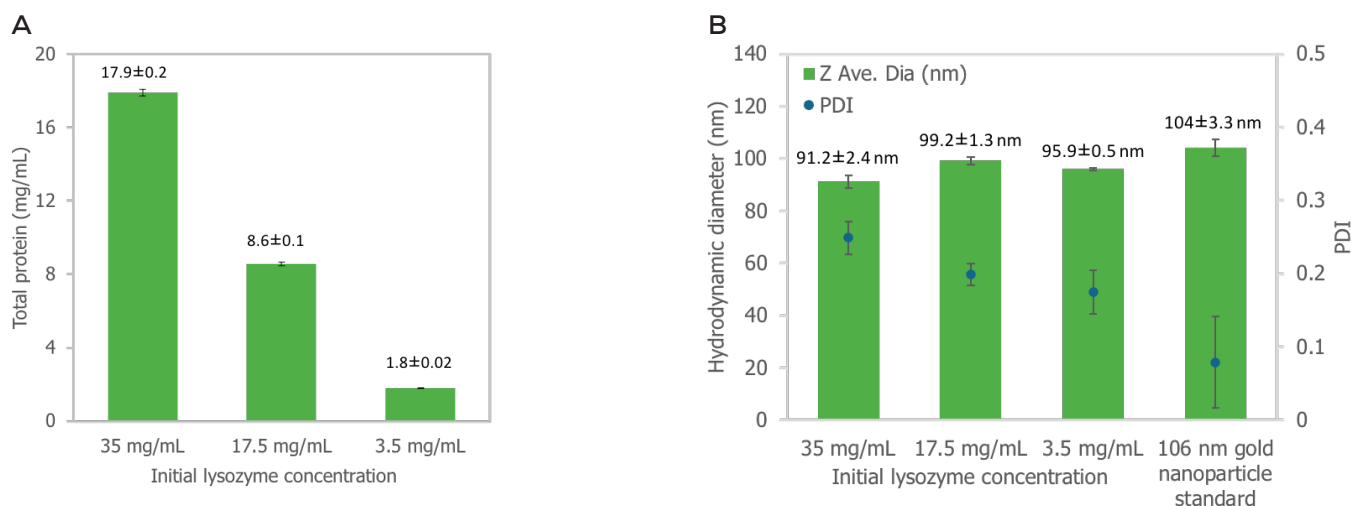


Figure 7: Stunner’s total protein quantifications (A) and size and polydispersity (B) of lysozyme-loaded LNPs were highly reproducible for varying initial lysozyme concentrations. Error bars are  $\pm 1$  standard deviation (SD).

protein. Each of the LNPs final total concentration was roughly 50% of the initial concentration. The size of the 3 LNPs varied between 90–100 nm, depending on the sample (Figure 7B). The PDIs for all 3 samples were  $>0.2$ , indicating that they were all highly polydispersed. To validate DLS size for the protein-LNPs, a 106 nm hydrodynamic diameter gold nanoparticle standard had an average hydrodynamic diameter of  $104 \pm 3.3$  nm by Stunner's DLS and an average PDI of 0.08.

## Custom Nanoparticle

Nanoparticles can get quite complicated with multiple, specialized components and diverse payloads, including small molecules or other drugs. Teaching Stunner to recognize and understand novel spectra is a snap with the Stored Spectra feature. Just make a dilution series and Stunner handles the rest. Once a payload spectrum is in the database the Custom Nanoparticle app can use it to deconvolute raw absorbance spectra into the contributions from

the payload, turbidity and particle & buffer components, just like the built-in LNP apps (Figure 8).

Creating drug-loaded nanoparticles requires careful optimization of manufacturing conditions.<sup>5</sup> Characterizing the products takes time, especially when determining drug content and nanoparticle size separately. Stunner offers a quick, accurate, and precise way to quantify total drug content and nanoparticle size simultaneously for any test condition. Mixes of doxorubicin (DXR)-loaded and control empty liposomes have different lipid:DXR ratios and DXR concentrations. Stunner's DXR quant is reproducible and accurate, as shown by the linear regression of measured DXR to target DXR, based on the manufacturers CoA, with an  $R^2 > 0.99$ , slope near 1, and y-intercept close to 0 (Figure 9A).

The DXR-loaded liposomes were slightly larger and had lower PDI than the control liposomes (Figure 9B). As the percent of control vs. DXR-loaded liposome increased, Stunner's average hydrodynamic diameter decreased and PDI increased. These results were reproducible with the particle size CVs size typically  $<2\%$  and PDI SDs  $<0.02$ .

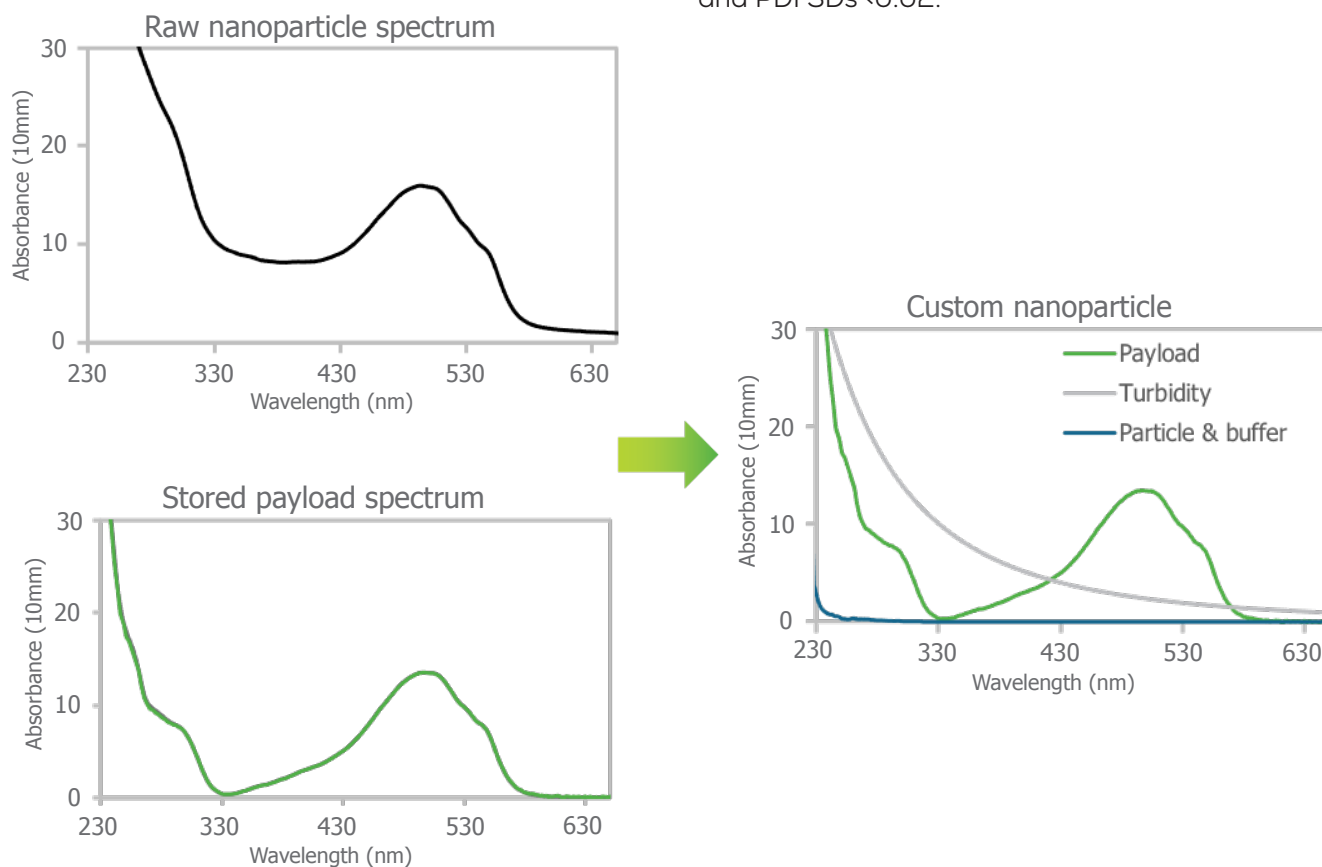


Figure 8: Storing spectra to Stunner's database lets you build reference analytes. The Custom Nanoparticle app uses those reference analytes to deconvolute a raw absorbance spectrum into the payload, turbidity and particle & buffer components. The payload absorbance can quantify the LNP payload directly – without complex workflows or particle disruption.

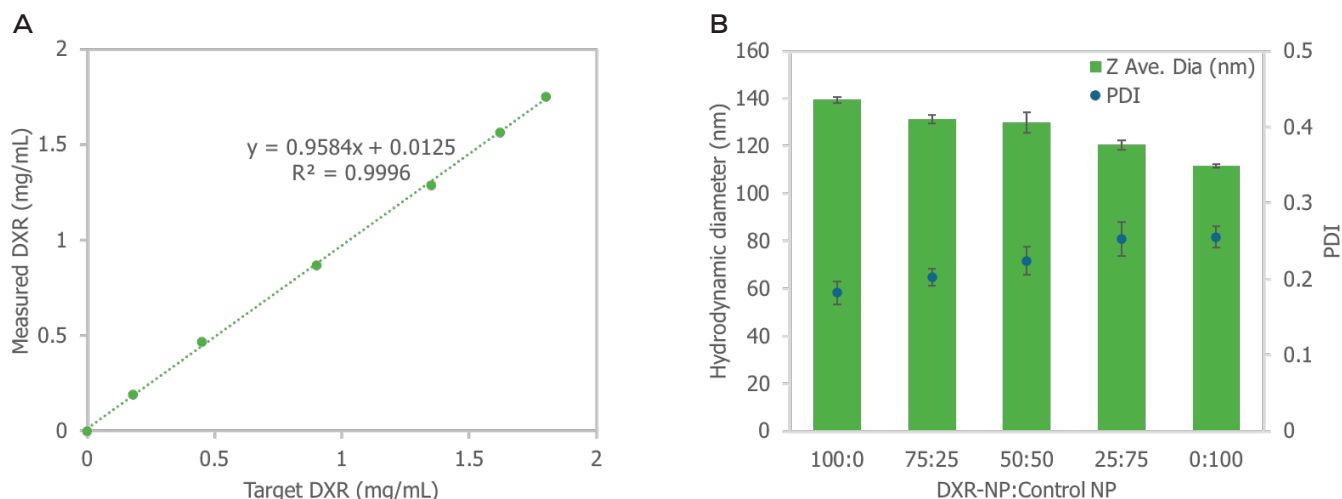


Figure 9: Stunner's quantification of mixes of DXR-loaded and empty control NPs showed strong agreement between target and measured DXR concentration (A). Stunner's size CVs were typically <2% and the polydispersity standard deviations were <0.02 (B). Error bars are  $\pm 1$  standard deviation (SD).

## Conclusion

Stunner is the one-and-only platform that combines UV/Vis and DLS for high-throughput, non-destructive quantification of payloads and lipid concentrations alongside sizing of nanoparticles. Crazy simple UV/Vis measurements give reagent-free, standard-free and hassle-free quants. A suite of LNP applications, along with the Custom Nanoparticle app, helps you analyze your nanoparticle, no matter what's loaded inside: RNA, DNA, protein or small molecules. Deconvolution of a nanoparticle's absorbance spectrum into its components lets you cut straight to your payload concentration despite high turbidity or lipid & buffer absorbance. Free yourself from complicated disruption workflows, costly dyes, wasteful standard curves and one-at-a-time DLS sizing with Stunner's low-volume, high-throughput nanoparticle characterization.

## References

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