# Have it all with protein quant & sizing on Stunner

## Introduction

Protein quantification and biophysical characterization of size and molecular weight are the first steps in understanding what's going on with your sample at every stage of biologic development and manufacturing. Many of the tools scientists use for this purpose take up too much time and a lot of precious sample. High-throughput, low volume characterization is therefore critical to evaluating proteins and speeding up pipelines.

Assays to quantify protein concentrations often rely on difficult-to-track dilutions, costly dyes, or complicated standard curves. Such methods introduce biases that can impact the accuracy and precision of the results.

Diameter and polydispersity are critical attributes for determining the quality of a protein preparation to know if aggregation has struck. Dynamic light scattering (DLS) is the classic method for non-invasively determining these parameters but many DLS instruments are cuvette-based and are stuck measuring one sample at a time. In addition, many of these instruments require significant dilution to get accurate, reliable results.

Stunner is the only platform that combines high-throughput UV/Vis with rotating angle DLS (RADLS) and multi-angle static light scattering (MALS) to give you protein concentration, molecular weight, particle count, size and polydispersity, on a single 2 µL sample (Figure 1). All of Stunner's RADLS and UV/Vis data is measured in a 96 well plate-based format and it takes under 2 minutes per sample without the need for dilutions, standards, or dyes.

This app note illustrates how to use Stunner to determine the size and polydispersity of proteins and particles in solution, quantify protein concentrations, measure protein molecular weights, and check for aggregation.


Figure 1: Stunner is the only system that pulls together UV/Vis, RADLS and MALS data on the same 2 µL sample

For a deep dive into the details of RADLS, see the Tech Note "Dive deep into Stunner's light scattering".

## Methods

Stock solutions of 10 g/L polystyrene 41 nm and 202 nm NIST™ traceable size standards (Thermo Fisher Catalog #3040A and 3200A) were diluted 10- and 50-fold, respectively, in water and measured 20 times in Stunner. The 20:1 mixture was made by diluting the 41 and 202 nm spheres in water to a final concentration of 0.24 and 0.012 g/L, respectively, then measured in triplicate.

Lyophilized powders of bovine serum albumin (BSA), conalbumin, human immunoglobulin G (IgG) and ovalbumin (MilliporeSigma) were reconstituted in phosphate-buffered saline (PBS). All of these stock solutions and a solution of bovine RNaseA (Qiagen) were diluted to 5 mg/mL in PBS. All of the working solutions were then filtered through 0.1 or 0.02 µm syringe filters and measured in quadruplicate.

A monoclonal antibody (mAb1) was diluted to 10 mg/mL in 5 mM sodium succinate, 60 mM trehalose, pH 5.0 and filtered through a 0.1 µm syringe filter. An aliquot from the mAb1 solution was taken and heated to ~80 °C for 15 minutes to in-

duce aggregation. The aggregated mAb1 was then combined with the non-aggregated sample in a ratio of 99:1 and 9:1 to make 99% and 90% mAb1 samples, respectively. Samples were measured on Stunner in octuplicate. Another aliquot of mAb1 was heated at ~80 °C for 30 minutes and samples were taken at 0 seconds, 10 seconds, 30 seconds, 1 minute, 3 minutes, 5 minutes, 10 minutes, and 30 minutes. These time course samples were measured on Stunner in quadruplicate.

Protein concentration, particle concentration, molecular weight, Rayleigh ratio, hydrodynamic size and polydispersity were assessed using the Stunner "Protein (Turbidity) + RADLS" application. NIST™ traceable size standards were measured using the "Concentration + Sizing" application. PBS, water, or succinate buffer (as detailed above) were used as a blank, where appropriate. A buffer viscosity and refractive index of 1.002 cP and 1.334, respectively, at 20 °C were used for water and PBS while values of 1.105 cP and 1.336 were used for succinate buffer. The default acquisition settings of 7 angles, 5 acquisitions, and 1 second each were used for RADLS with the software's automatic angle selection and outlier exclusion. Table 1 contains the E1% values used for each protein.

## Results

### Sizing so close you can shave with it

Easy, precise sizing of nanoparticle standards makes any analytical method validation for biologics simpler. Stunner's RADLS measures multiple angles, so it can see large particles that would otherwise be hard to detect. Plus, RADLS optics self-optimize to automatically adjust for the best beam overlap for every measurement and sample. That means Stunner's RADLS gives really precise DLS reads and makes it easy to determine the size of particles in solution from just 2 µL of sample.

A pair of polystyrene NIST™ traceable size standards of 41 and 202 nm were measured on Stunner and had CVs of 2.9 and 2.1%, respectively (Figure 2). They also had PDIs <0.1, characteristic of monodisperse particles, meaning a homogenous and uniform population of particles in solution. Mixing the 41 and 202 nm size standards in a 20:1 ratio shows what happens when you measure a polydisperse, heterogenous sample: the Z-average diameter was between the nominal size of the spheres, and the PDI increased to >0.2.

Values from the cumulants method of analyzing RADLS data, like Z-average diameter and PDI, are great for telling if a sample is uniform or not and for telling the size of monodisperse particles. However, it leaves a lot to be desired when looking at polydisperse samples. Regularization analysis, on the other hand, is much more useful when looking at non-uniform particle distributions. This method provides a best-fit for the size distribution of particles present in a heterogenous sample.

Stunner's robust regularization analysis gives highly reproducible distributions of the 20:1 mix of the size standards (Figure 3). The overlay of intensity distributions (left, green) from triplicate

| Protein | Extinction coefficient (E1%) |
|---|---|
| BSA | 6.67 |
| Conalbumin | 11.6 |
| IgG | 13.7 |
| Ovalbumin | 7.5 |
| RNaseA | 8.99 |

Table 1: Extinction coefficients (E1%) of proteins.

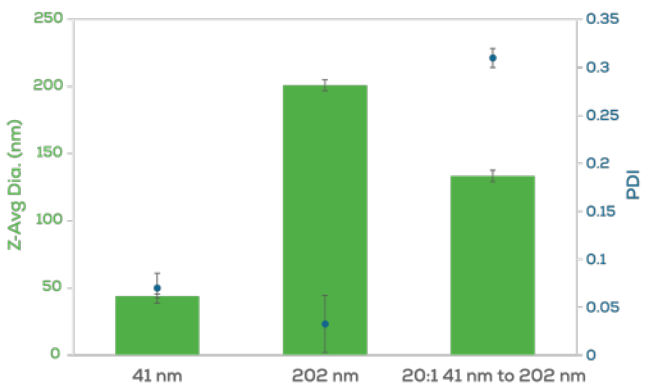


Figure 2: Z-average diameter (green, left y-axis) and polydispersity index (PDI; blue, right y-axis) of 41 and 202 nm polystyrene NIST™ traceable size standards and a 20:1 mix of the standards. Error bars represent 1 standard deviation.

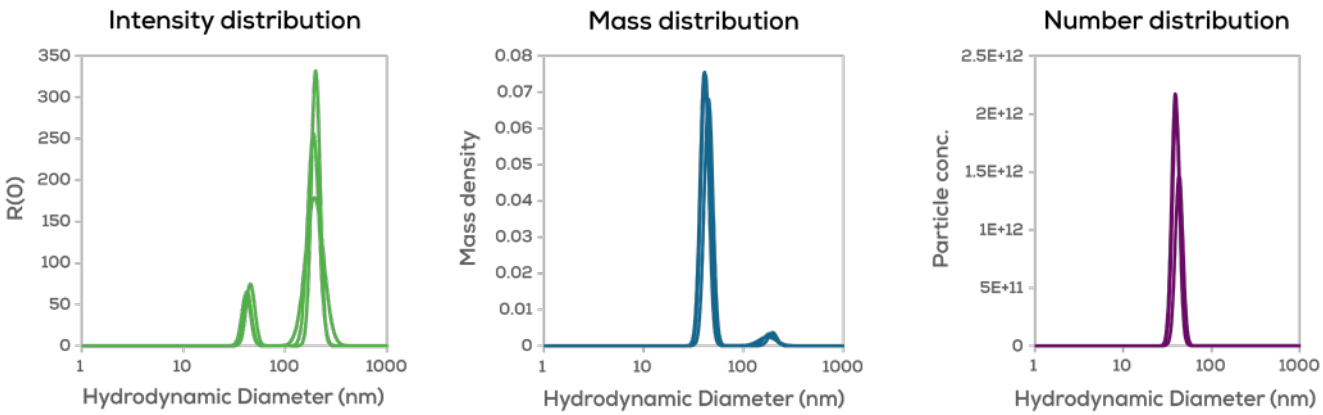


Figure 3: Overlaid triplicate intensity (green, left), mass (blue, middle) and number (purple, right) distributions of a 20:1 mix of 41 and 202 nm polystyrene NIST™ traceable size standards in water.

measurements shows much higher peaks at 200 nm than 40 nm, simply because larger particles scatter light more intensely than smaller ones (light scattering increases with the 6th power of size). The mass distributions from the same measurements (middle, blue) show the inverse peak height ratio because the smaller particles were a larger fraction of the overall mass density of particles in the solution. The number distributions (right, purple) show only a single peak at ~40 nm since the smaller particles have a much higher particle concentration than the larger ones.

## Protein quant & sizing will never be the same

Since protein quant by UV/Vis absorbance doesn't rely on standard curves it's extremely precise, accurate, and reliable. Five proteins were prepared to a target concentration of 5 mg/mL in PBS (Figure 4).

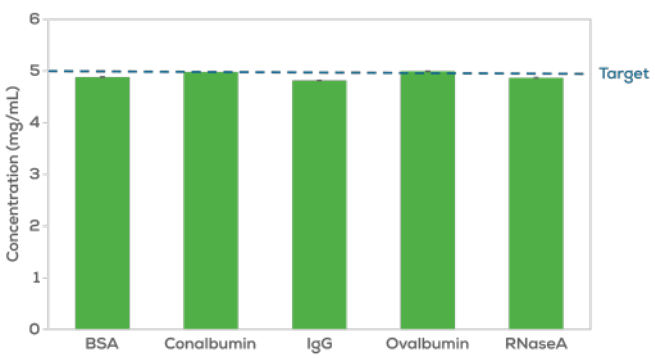


Figure 4: Protein concentration by A280 of 5 mg/mL BSA, conalbumin, IgG, ovalbumin, and RNaseA in PBS. Error bars represent standard deviation.

The measured concentrations for all 5 were within 2% of the target value and had less than 1% CVs.

True head-to-head comparisons of proteins demand more than just their concentrations. They also require real sizes and particle counts. Stunner collects multi-angle static light scattering (MALS) intensities along with its RADLS measurements. Each of these scattering intensities are proportional to particle number – however, these measurements also are impacted by the scattering angle. By combining the MALS data of the various angles, Stunner can get angle-independent results for biologics of any size. Consequently, Stunner's intensity, mass, and number distributions are real, not normalized, and they can be used to make sample-to-sample comparisons.

The scattering intensity of a 5 mg/mL sample of IgG in PBS is significantly higher than ovalbumin at the same concentration (Figure 5, top, light vs dark green), since the molecular weight of IgG is about 3 times higher than that of ovalbumin. When looking at the mass distribution (Figure 5, middle, blue) the peaks are approximately the same height, despite the proteins being different sizes, because they have the same concentration, or overall mass in solution. Ovalbumin has a higher particle count, or molarity, than IgG at the same mass concentration – therefore its peak in the number distribution (Figure 5, bottom, dark purple) is much higher.

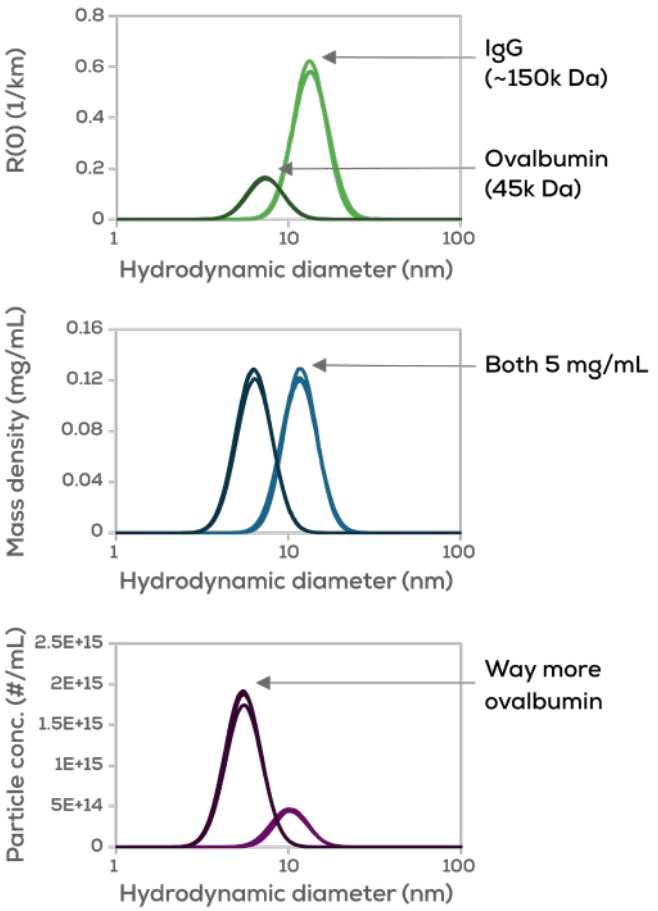


Figure 5: Overlaid triplicate intensity (green, top), mass (blue, middle) and number (purple, bottom) distributions of 5 mg/mL IgG (light colors) and ovalbumin (dark colors) in PBS.

## Molecular weights, no waiting

Stunner uses the light scattering intensities, expressed as a Rayleigh ratio, R(θ), at 7 angles to extrapolate what the intensity would be at 0°, or R(0). A scattering angle of 0° is the theoretically "best" angle for the highest intensity of scattered light from the widest range of particle sizes, but measuring at this angle is technically impossible because the detector would be looking directly into the laser. Depicting R(0) extrapolation graphically using a Guinier plot of IgG, conalbumin, ovalbumin and RNaseA in PBS shows another property of the Rayleigh ratio: it's proportional to molecular weight (Figure 6). Using R(0) you can make reasonably accurate molecular weight determinations of uniform, purified proteins using Equation 1:

$$R(0) = KcM$$

where K is the optical contrast constant, which primarily depends on the refractive index increment of the analyte (dn/dc), c is the concentration of the protein, and M is the molecular weight.

The results for the 4 proteins shown in Figure 6, along with BSA, are in Table 2. (The calculations used an approximated dn/dc for all five proteins.)

## Putting it all together

Nailing down biologic quality needs every tool one has at one's disposal. Stunner delivers concentration, diameter and molecular weight in less than 2 minutes per sample, which is great for quickly detecting aggregates in a protein sample. When used comparatively, it can even approximate the extent of aggregation.

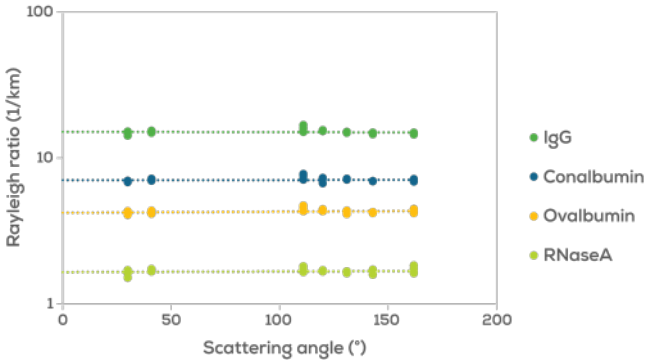


Figure 6: Guinier plot of Rayleigh ratio (R) vs scattering angle (°) for 5 mg/mL IgG (green), conalbumin (blue), ovalbumin (yellow) and RNaseA (light green) in PBS measured in quadruplicate with extrapolation of R(0).

| Protein | Reference MW (kDa) | Stunner MW (kDa) |
|---|---|---|
| BSA | 67 | 70±1.3 |
| Conalbumin | 76 | 64±0.6 |
| IgG | 150 | 150±1 |
| Ovalbumin | 45 | 39±0.5 |
| RNaseA | 14 | 17±0.4 |

Table 2: Reference and Stunner-determined molecular weights of 5 proteins. The MWs were calculated from quadruplicate Stunner measurements with the equation MW= R/(K*c) where K is the optical contrast constant for the different proteins (approximated using the same dn/dc for all samples) and c is the concentration determined by UV/Vis absorbance.

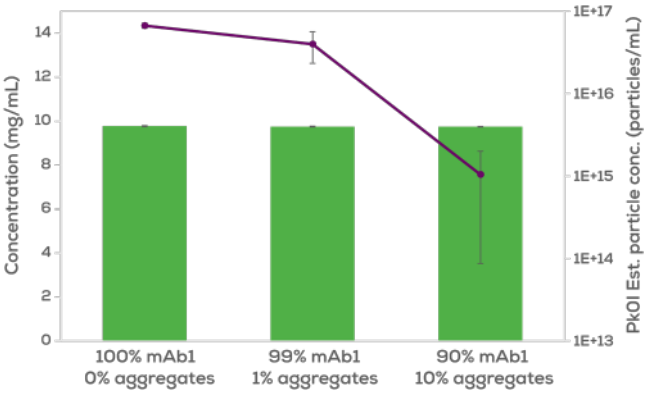


Figure 7: Mass concentration (green, left y-axis) by UV/Vis absorbance and particle concentration (purple, right y-axis) of 0%, 1%, and 10% aggregated 10 mg/mL mAb1 in sodium succinate buffer.

Monoclonal antibody samples with 0%, 1%, and 10% aggregates had identical concentrations by UV/Vis but decreasing particle concentrations by RADLS (Figure 7). Protein aggregates are much larger, both in terms of molecular weight and diameter, than the monomers and as such fewer individual particles are required to reach the same mass concentration. The intensity and mass distributions of the mAb samples reflect this (Figure 8). Intensity and mass distributions from eight replicate RADLS measurements of the 0% aggregated mAb sample show single, well-defined, overlapping peaks. Distributions from a 1% aggregated sample show 2 peaks in the intensity distributions but only 1 peak in the mass distribution. In a 10% aggregated sample, the 2nd peak is visible in both the intensity and the mass distributions.

Stunner can deliver quantitative protein mass concentrations and detect aggregation. It can also semi-quantitatively determine the extent of aggregation when comparing samples to each other.

## Don't stress about it

Biologics undergo a battery of forced degradation studies during the development process. Stresses of all kinds are used to induce protein damage, including temperature, pH, and mechanical. Stunner's UV/Vis and RADLS technology give a quick snapshot of what's going on during a stress test by assessing aggregation at sampled time points. For example, even though the UV/Vis concentration of mAb1 did not change during a heat stress time course, the hydrodynamic diameter of the major peak in the mass distribution increased sharply after 10 minutes (Figure 9). By 30 minutes, the protein had completely aggregated.

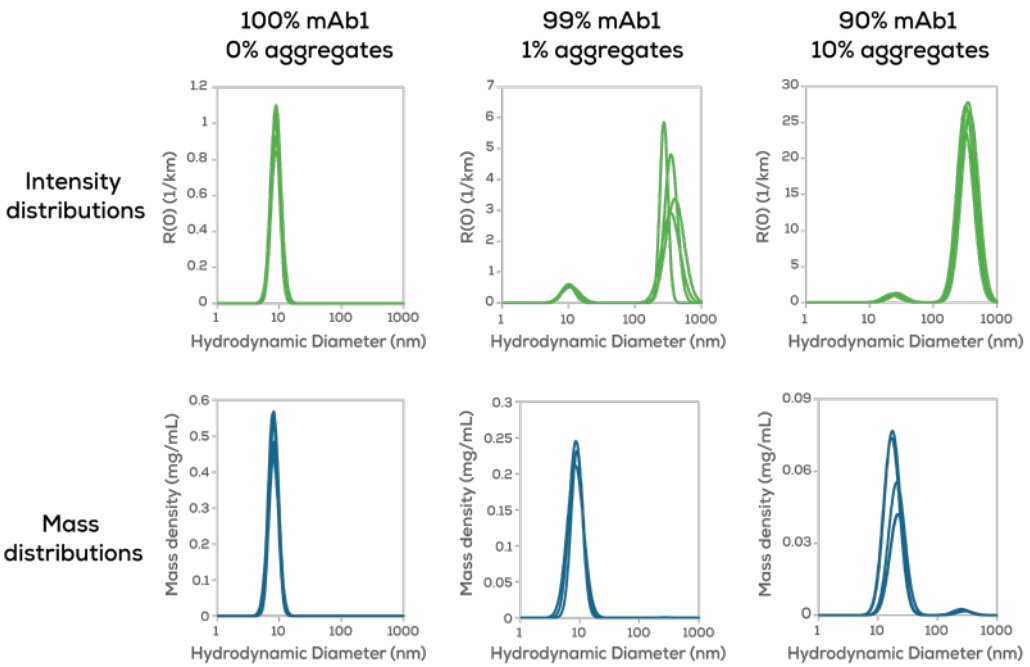


Figure 8: Overlaid octuplicate intensity (green) and mass (blue) distributions of 0% (left), 1% (middle) and 10% (right) aggregated 10 mg/mL monoclonal antibody in sodium succinate buffer.

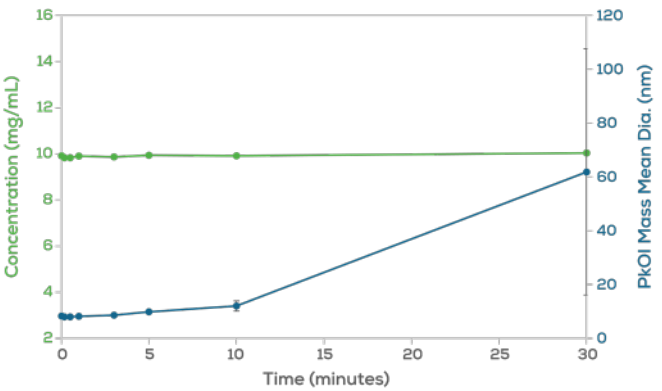


Figure 9: UV/Vis concentration (green, left y-axis) and mean diameter of the peak of interest (blue, right y-axis) of mAb1 heated to 80 °C. Quadruplicate samples were taken at the indicated time points and measured in Stunner. Error bars represent standard deviation.

## Conclusion

Stunner makes getting high-throughput, low volume answers to biophysical characterization questions simple. RADLS and UV/Vis data gives precise results on protein concentration, molecular weight, aggregation, size and polydispersity. Real intensity, mass, and number distributions let you compare samples head-to-head to determine the extent of aggregation in protein samples, be they stressed or simply unknown. With 2 µL and in less than 2 minutes, Stunner delivers dilution-free, standard-free, hassle-free protein characterization on any biologic you throw at it.

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