Uncle sets a new benchmark for protein characterization

Introduction
The monoclonal antibody reference material distributed by the National Institute of Standards and Technology (NIST) has been extensively characterized by many researchers, and has become an excellent standard for the biopharmaceutical industry. The large body of data that has been collected and shared on this molecule is invaluable for verifying analytical methods across research and process development groups. In this application note, we describe NISTmAb data collected on the Uncle platform that demonstrates the versatility, reliability and ease-of-use of the instrument.

Uncle is an all-in-one stability platform that enables 12 different applications with one instrument (Figure 1). Fluorescence, static light scattering (SLS) and dynamic light scattering (DLS) detection methods are used to characterize protein stability. Temperature control (15–95 °C) and sealed samples provide greater flexibility in how characterization can be performed. Multiple measurements, such as thermal melting, aggregation and sizing can be collected in a single experiment with the same set of samples. Samples are loaded into Unis, low volume, multi-well quartz cuvettes that let you run up to 48 samples at a time. Uncle makes it easy to thoroughly characterize more biologics and formulations earlier than before.

Methods
Reference Material 8671, or NISTmAb, was obtained at 10 mg/mL in 12.5 mM L-histidine, 12.5 mM L-histidine HCl (pH 6.0). Dilutions of the antibody were made to 0.5, 1, 2, 4, 6 and 8 mg/mL in the same formulation buffer. The 10 mg/mL stock solution was also exchanged into 2 other formulations: 30 mM citrate, 200 mM NaCl (pH 5.0) and 30 mM sodium phosphate, 200 mM NaCl (pH 8.5). The same dilution series were prepared for the two additional formulations. All protein concentrations were verified with A280 measurements collected on a Lunatic.

Sizing and Polydispersity
Nine µL of each sample at concentrations ranging from 2 mg/mL to 10 mg/mL in the Histidine buffer were pipetted into 13 wells of a Uni and run on Uncle, with 10 x 10s acquisitions. The viscosity of the sample at 25 °C was assumed to be 0.9 cP and the refractive index was approximated to be 1.33.

Thermal Stability and Aggregation with DLS
Nine µL of NISTmAb at 0.5 mg/mL and 1 mg/mL in three different formulation conditions were loaded into Unis in triplicate and run with a thermal ramp from 15 °C to 95 °C and a ramp rate of 0.3 °C/minute on Uncle. Uncle Analysis software calculated the T_m using the first derivative of the barycentric mean (BCM) of fluorescence intensity, and calculated the T_agg at 266 and 473 nm. DLS measure-
ments were collected before and after the thermal ramp, with 4 x 5s acquisitions.

**B<sub>22</sub> and k<sub>D</sub>**

Nine µL of NISTmAb at 2, 4, 6, 8 and 10 mg/mL in each of the 3 formulation buffers were loaded in triplicate, along with one well containing each buffer for blank subtraction. Samples were run with the B<sub>22</sub> and k<sub>D</sub> application on Uncle with 4 x 5s acquisitions. Toluene was used as a reference intensity standard for B<sub>22</sub> measurements. HPLC-grade toluene was loaded into eight wells of a Uni, and the average scattering intensity from the DLS acquisition was used by Uncle Analysis software in conjunction with the blank-subtracted light scattering intensities from the protein samples.

**Results**

The hydrodynamic diameter of NISTmAb in its supplied formulation buffer was measured by DLS using the Sizing and Polydispersity application on Uncle. As demonstrated by a representative sample at 4 mg/mL, all samples yielded one monodisperse peak at ~10 nm, which is the expected size for a monoclonal antibody (Figure 2). The average values from 13 samples, ranging in concentration from 2 to 10 mg/mL, measured 10 times each (n=130) are shown in the inset table. The Sizing and Polydispersity results indicate that there was no aggregation after thawing and handling of the NISTmAb samples.

To evaluate the thermal stability of NISTmAb, the intrinsic fluorescence of the protein was measured under heat stress. Over a thermal ramp from 15 to 95 °C, the protein was found to undergo three distinct transitions, at 67.3, 80.0, and 90.3 °C (Figure 3). These align well with the results from the DSC thermogram published by NIST, where the antibody at the same concentration also showed three thermal transitions, at about 69, 83, and 94 °C (Reference 1). T<sub>m</sub> values measured by fluorescence or DSF tend to be lower than values measured by DSC, as changes in fluorescence due to conformational changes of the protein are more sensitive than those measured by heat exchange.

The onset of aggregation, or T<sub>agg</sub>, can be obtained at the same time and from the same set of samples as T<sub>m</sub>. By collecting static light scattering (SLS) data at two different wavelengths simultaneously throughout a thermal ramp, information about the formation of both small and larger aggregates can be easily obtained. NISTmAb was exchanged from its supplied formulation buffer into two additional formulation conditions at lower and higher pH, with added salt. The goal of these experiments was to see what effect changing the formulation would have on the tendency of the molecule to aggregate. Interestingly, whereas the mAb in its sup-

**Figure 2:** The intensity distribution of a representative sample of NISTmAb showing a single intensity peak at about 10 nm. The calculated hydrodynamic diameter and PDI value, as well as the mean values from 13 samples at a range of concentrations are shown.

**Figure 3:** The thermal melting curve of NISTmAb over a 15 to 95 °C temperature ramp, where 3 distinct transitions are detected.
plied formulation showed no significant increase in scattering intensity at 266 nm across the entire thermal ramp, the two other formulations both displayed drastically different curves (Figure 4). SLS performed simultaneously at 473 nm (not shown) yields a similar result. In both formulations, the scattering intensity sharply increased between 73 and 74 °C. In Citrate buffer at pH 5, the intensity remained high through the end of the ramp, whereas in Phosphate buffer at pH 8.5, the intensity dropped back down to almost 0 at around 85 °C. This could indicate the precipitation of particles out of the scattering volume, or the disassembly of aggregates at higher temperatures.

Uncle also enables the collection of DLS data at the beginning and end of a thermal ramp with no extra sample or set-up time required. In this case, DLS can help to distinguish between the different aggregation possibilities described above. To gain a more quantitative picture of aggregation, sizing and polydispersity data was acquired at 15 °C and 95 °C for the NISTmAb in each formulation. The DLS results confirm the analysis of the SLS data. NISTmAb in the supplied Histidine buffer showed very little change in hydrodynamic diameter after being subjected to the thermal ramp, confirming that little to no aggregation occurred. Additionally, the PDI value at the end of the thermal ramp was still less than 0.250, indicating that the sample remained monodisperse (Figure 5).

In contrast, the mAb in Citrate buffer at pH 5 with salt showed a dramatic increase in size after the thermal ramp, with a final size measured at over 260 nm (Figure 6). At 95 °C the PDI value increased by an order of magnitude, indicating that the sample was polydisperse and contained significant aggregate content.

NISTmAb in Phosphate buffer at pH 8.5 with salt showed an even larger change after the thermal ramp, with a final size of over 2700 nm (Figure 7). Similar to the Citrate buffer results, the PDI value increased by an order of magnitude, indicating that the sample was polydisperse and contained

![Figure 4](image1.png)  
Figure 4: The thermal aggregation curves of NISTmAb in three different formulations over a 15 to 95 °C temperature ramp.

![Figure 5](image2.png)  
Figure 5: Initial (blue) and final (green) DLS measurements for NIST mAb in its supplied formulation buffer. The graph shows the intensity distribution for each sample, and the inset table displays the calculated hydrodynamic diameter and polydispersity index.

![Figure 6](image3.png)  
Figure 6: Initial (blue) and final (green) DLS measurements for NIST mAb in a sub-optimal formulation buffer. The graph shows the intensity distribution for each sample, and the inset table displays the calculated hydrodynamic diameter and polydispersity index.

![Figure 7](image4.png)  
Figure 7: Initial (blue) and final (green) DLS measurements for NIST mAb in its supplied formulation buffer. The graph shows the intensity distribution for each sample, and the inset table displays the calculated hydrodynamic diameter and polydispersity index.
significant aggregate content. The presence of large aggregates at the end of the thermal ramp provides more quantitative confirmation of the SLS data and indicates that particles might have been starting to sediment.

Two well-established methods for characterizing protein stability and aggregation propensity, particularly when comparing different formulation conditions, are the diffusion interaction parameter \(k_D\) and the second virial coefficient \(B_{22}\). Uncle makes both of these measurements and resulting analysis quick and easy. To measure both parameters simultaneously, a dilution series of the protein in each condition was made, where ~10 mg/mL was the highest concentration used, and ~2 mg/mL was the lowest. The measured concentrations of each sample were used for the calculations.

Negative \(k_D\) and \(B_{22}\) values indicate conditions where the protein favors self-association over complete solvation, while positive values indicate repulsive interactions between protein molecules, which is a more desirable scenario for developing a therapeutic molecule. In the optimal Histidine buffer, NIST mAb yielded positive \(k_D\) and \(B_{22}\) values (Figure 8 and 9). The sign and magnitude of the \(k_D\) and \(B_{22}\) values measured on Uncle are in agreement with what has previously been report-
Conclusion

With very little hands-on time, Uncle was able to generate reliable data that agreed with previously reported results on the reference standard molecule, NISTmAb. The sizing and polydispersity of the material was determined by Uncle and was in agreement with previous DLS experiments. In addition, the thermal stability profile of the molecule was found to be comparable with results obtained by DSC. Finally, we demonstrated how Uncle can be used in formulation screening and optimization by providing additional, novel data on thermal aggregation and aggregation propensity. The versatility of the Uncle platform can help researchers reduce their risk when developing biotherapeutic molecules, by providing methods for obtaining stability and protein characterization data earlier in the process, with less sample.

References