Power smarter formulation screening with Aunty

Introduction

Increasingly complex protein therapeutics require equally complex formulations to maintain safety, stability and efficacy. Screening those conditions is often slow, sample-intensive, and fragmented across multiple assays. Even when applying a Design of Experiment approach (DoE), traditional tools typically generate only a single readout per run, limiting how much insight you gain from each experiment.

Aunty changes that by enabling true high-throughput, multi-parameter stability screens (Figure 1). Using SBS-format plates, Aunty measures conformational and colloidal stability of up to 96 samples in the same experiment to identify melting temperature (T_m), onset of unfolding (T_{onset}), onset of aggregation (T_{agg}), and temperature of size change (T_{size}). Each well only uses 8 µL of sample and Aunty measures all 96 samples every minute of the experiment, so you'll extract more insights per sample and per run.

Full-spectrum differential scanning fluorescence (DSF) detection in Aunty gives you the flexibility to use intrinsic or dye-based emissions to determine T_ms, while static and dynamic light scattering (SLS & DLS) run in parallel to spot aggregates before, during, or after heating. The same platform can also assess intermolecular interactions using SLS & DLS with a dilution series, helping predict aggregation propensity across formulations. Combine Aunty and its built-in visualization tools with DoE, Bayesian modeling, or AI workflows to enable faster identification of optimal formulations with fewer experiments and less material.

This app note shows how to use Aunty's *T_m & T_{agg} with sizing* and *Colloidal stability* applications with full-spectrum DSF, sensitive SLS, reliable DLS, and highly accurate temperature control to determine key stability metrics in a DoE formulation study.

Figure 1: Aunty is the world's first flexible, automation-friendly, fit-for-purpose, plate-based thermal stability platform.

Methods

Three monoclonal antibody (mAb) biosimilars (Syd Labs, Hopkinton, MA) were diluted to 10 mg/mL with 10 mM histidine/histidine-HCl, pH 5.5. Initial formulations are shown in Table 1. Carbonic anhydrase isozyme II from bovine erythrocytes (Sigma-Aldrich, Inc., St. Louis, MO) was dissolved in deionized water to 10 mg/mL. All four proteins were then buffer exchanged into the formulation to be tested (without polysorbate 80) using a ultrafiltration/diafiltration approach with Unfilter 96, 10 and 30 kDa for carbonic anhydrase and the mAbs, respectively, and default parameters for protein solutions from 0.5–50 mg/mL.

A fractional-factorial design with two levels and 5 variables was used at resolution 3 with the assumption that two-factor interactions would be much smaller than the main effects.[1] Initial and final protein concentrations were checked in quadruplicate on Stunner with buffer blanks. PS80 or water was added to achieve the final formulations shown in Table 2.

All 32 samples (4 proteins x 8 formulations) were diluted to 2 mg/mL and 8 µL were heated from 15 to 95 °C, in triplicate, at a rate of 1 °C/minute using Aunty's *T_m & T_{agg} with sizing* application, with automatic settings throughout; the experiment took an hour and 20 minutes and a single plate.

Protein	Buffer	pH	Excipients
50 mg/mL adalimumab biosimilar	Citrate-phosphate	5.2	6.2 mg/mL NaCl, 12 mg/mL mannitol, 1 mg/mL PS80
10 mg/mL rituximab biosimilar	Citrate	6.5	9 mg/mL NaCl, 0.7 mg/mL PS80
20 mg/mL trastuzumab biosimilar	Histidine	6	19.09 mg/mL trehalose, 0.09 mg/mL PS20
Carbonic anydrase isozyme II	Lyophilized	N/A	N/A

Table 1: Starting formulations of the 3 mAb biosimilars and carbonic anhydrase used in this formulation study.

	His (mM)	pH	Arg (mg/mL)	NaCl (mg/mL)	Sucrose (mg/mL)	PS80 (mg/mL)
Formulation 1	9.3	5.5	0	8.4	93.3	0
Formulation 2		5.5	9.3	0	0	0
Formulation 3		5.5	0	0	93.3	0.2
Formulation 4		5.5	9.3	8.4	0	0.2
Formulation 5		7	0	8.4	0	0
Formulation 6		7	9.3	0	93.3	0
Formulation 7		7	0	0	0	0.2
Formulation 8		7	9.3	8.4	93.3	0.2

Table 2: Fractional-factorial design matrix for formulation variables with level concentrations of histidine (His), arginine (Arg), sodium chloride (NaCl), sucrose, and polysorbate 80 (PS80).

Plate layout was randomized, per standard DoE practice. T_m values were identified at peaks in the differential of the barycentric mean (BCM) of the protein's intrinsic fluorescence emission (310–370 nm). T_{onset} values were determined from the increase in BCM. T_{agg} values were determined from the increase in SLS intensity and T_{size} values from the increase in z-average diameter determined by DLS.

For colloidal stability testing, a dilution series of each formulation was prepared with 5 concentrations per series. 8 µL of each sample were loaded in triplicate into Aunty plates. The *Colloidal stability* app was used, with buffer blanks, to determine B_{22} and k_D at 25 °C using 5x4 seconds DLS acquisitions and auto-attenuation. Each of the proteins required its own Aunty plate and 5 plates total were used for this experiment.

Results

T_m & T_{agg} with sizing

High-throughput thermal stability screens generate large, multi-parameter datasets, making efficient tools for data interpretation essential. Aunty's visualization tools enable quick comparisons of stability trends across proteins and formulations. For example, Aunty's heatmaps showed that carbonic anhydrase had lower melting temperatures than the mAbs (Figure 2A). The rituximab and trastuzumab biosimilars displayed lower T_ms in Formulation 1 (rituximab: A4, B5, G5; trastuzumab: A7, B8, G9). In contrast, the trastuzumab biosimilar exhibited higher T_ms in Formulation 6 (F7, H8, F9) and Formulation 7 (G7, D8, D9).

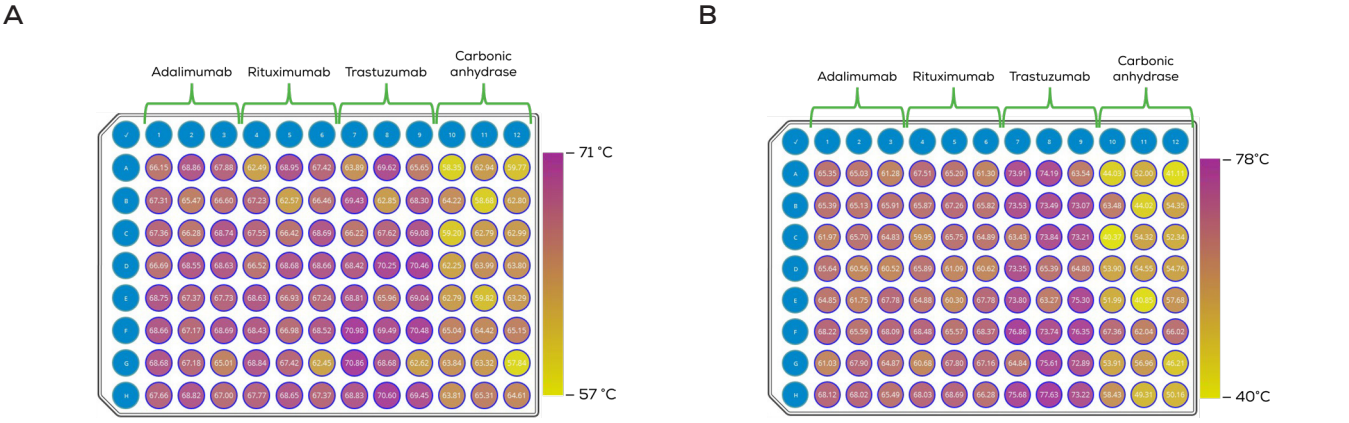

Figure 2: Heat maps of T_ms (**A**) with a minimum (yellow) at 57 °C and maximum (purple) at 71 °C and T_{agg}s (**B**) with minimum (yellow) at 40 °C and maximum (purple) at 78 °C of 3 mAb biosimilars and carbonic anhydrase in 8 formulations in triplicate. Replicates are in a randomized order.

Carbonic anhydrase had lower T_{agg}s than any of the mAbs, often lower than the T_m, indicating extreme aggregation propensity upon heating (Figure 2B). The trastuzumab biosimilar had the widest spread of T_{agg}s of all the tested molecules, with a low of ~63 °C in Formulation 3 and a high of ~77 °C in Formulation 6. These differences are readily visualized with Aunty's heatmaps, enabling rapid identification of the most and least stable conditions across the formulations.

Even though protein unfolding and aggregation are linked, aggregation can occur from native, degraded, partially unfolded, or completely unfolded states.[2] Formulations that stabilize the conformation of a protein can occasionally also drive aggregation. For example, the trastuzumab biosimilar in Formulation 7 had a higher T_m than in Formulation 1, but a lower T_{agg} (Figure 3A & B). Similarly, increases in the hydrodynamic diameter occurred in lower temperatures in Formulation 7. Together, these results show that while Formulation 7 improved the conformational stability of the trastuzumab biosimilar, it reduced colloidal stability.

Optimizing formulations requires balancing tradeoffs, and Aunty facilitates this by combining DSF, SLS, and DLS measurements in a single workflow so you can evaluate conformational and colloidal stability in parallel with fewer experiments and faster iteration.

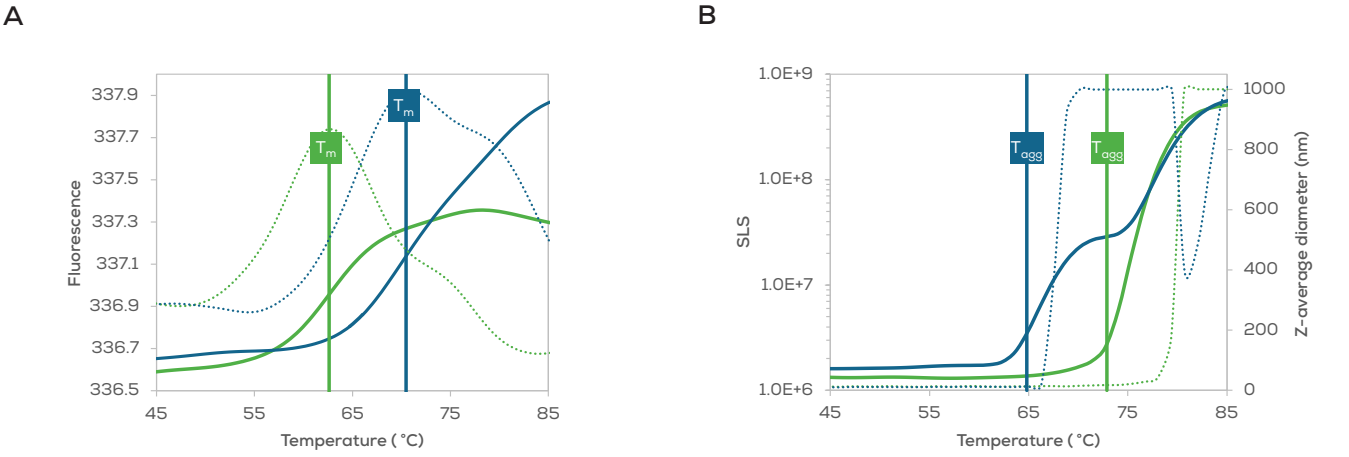

Figure 3: Trastuzumab biosimilar in Formulation 1 (green) and Formulation 7 (blue). Barycentric mean (solid) and differential (dashed) of the intrinsic protein fluorescence 310–370 nm with T_ms (**A**). SLS intensity (left y-axis, solid), hydrodynamic diameter (right y-axis, dashed), and T_{agg}s (**B**). Curves are representative of triplicates.

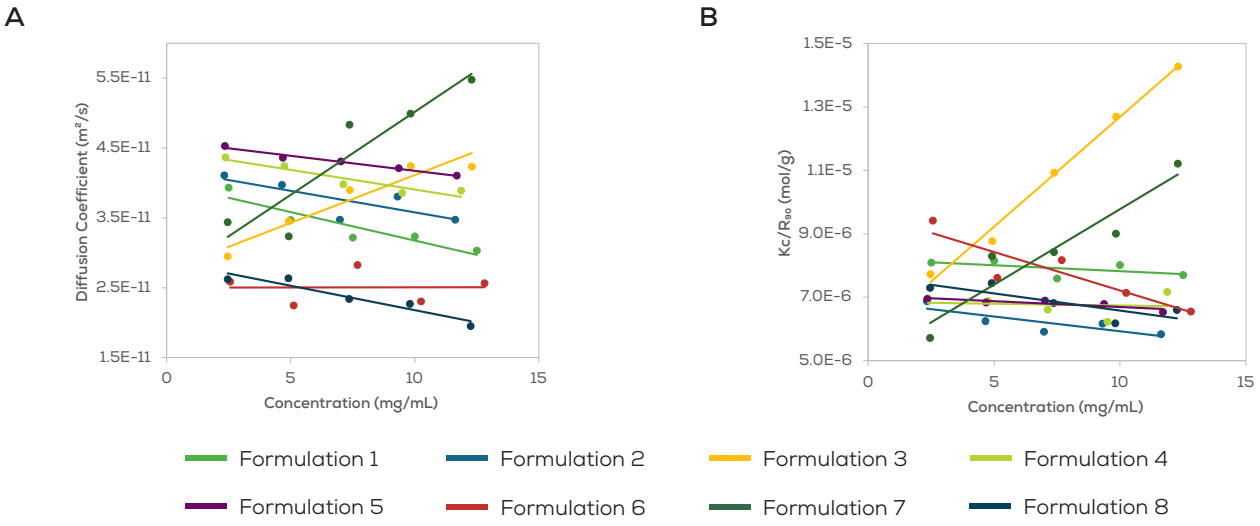

Figure 4: Average diffusion coefficient versus concentration (**A**) and Debye plot (**B**) of the rituximab biosimilar in Formulations 1–8 with linear regression.

Colloidal stability

Native-state aggregation propensity is a key attribute in formulation development and depends strongly on excipients and pH. The diffusion interaction parameter (k_D) and second virial coefficient (B_{22}) are commonly used to identify formulations with high aggregation risk and poor developability profiles.[3] Aunty's *Colloidal stability* app uses DLS to determine the diffusion coefficients of a dilution series and calculates k_D from the slope. At the same time, it uses SLS intensity versus concentration to calculate B_{22}. Negative k_D or B_{22} values indicate attractive molecular interactions and increased aggregation propensity, whereas positive values indicate repulsive self-interactions and improved stability.

While we collected k_D and B_{22} results on all the formulations, only the rituximab data is presented here in detail for representative purposes. Of the 8 tested formulations, the rituximab biosimilar had a strongly positive k_D and B_{22} value only in Formulations 3 and 7 (Figure 4). Other formulations had negative or near-0 k_D and B_{22}. The rituximab biosimilar experienced strong repulsive intermolecular forces in Formulations 3 and 7 and was therefore less prone to native state aggregation.

Excipient screening

As biologics increase in complexity, so do their formulations. Improving one stability metric or quality attribute often comes at the expense of another. Balancing these effects requires robust data sets and appropriate statistical analysis. Applying a DoE study design and Aunty's data export made it easy to identify which factors had significant impact on the conformational stability (T_m/T_{onset}), thermal aggregation (T_{agg}/T_{size}), and colloidal stability (k_D/B_{22}) from the 8 tested formulations (Table 3), accelerating identification of promising excipients and optimal pH.

All the tested mAbs had higher T_ms and T_{onset}s in pH 7 compared to pH 5.5. With isoelectric points (pIs) between 8.2 and 8.7, these molecules carry a greater net positive charge in the more acidic pH, which may reduce intramolecular stability and result in lower melting temperatures. Carbonic anhydrase had higher T_m, T_{onset}, and T_{agg} at pH 7, where it carries a net negative charge (pI = 5.4). This may have caused electrostatic repulsion between the molecules, improving thermal stability. The pH of the formulation had no significant impact on T_{agg} or T_{size} of the mAbs.

Arginine and NaCl increased T_{agg} and T_{size} but had a negative impact on k_D and B_{22}. PS80, a surfactant commonly added to reduce surface adsorption and

Protein	T_m/T_{onset}		T_{agg}/T_{size}		k_D/B_{22}	
	+	–	+	–	+	–
Adalimumab	pH 7	NaCl Sucrose	Arginine NaCl	PS80	PS80	Arginine
Rituximab	pH 7 PS80	NaCl Sucrose Arginine	Arginine NaCl	PS80	PS80	Arginine NaCl
Trastuzumab	pH 7 Arginine	Sucrose NaCl	Arginine NaCl	PS80	PS80	NaCl Arginine
Carbonic anyhydrase	Arginine pH 7	Sucrose NaCl	Arginine pH 7	Sucrose	No significant factors	

Table 3: Excipients which significantly increased (+ columns, green) or decreased (- columns, red) the indicated stability metrics for each of the proteins, based on regression analysis. Within a cell, the excipients are ordered by the relative magnitude of their effect.

denaturation at air-water interfaces[4], decreased T_{agg} and T_{size} but improved k_D and B_{22} . Although all 4 parameters relate to aggregation, they describe different mechanisms: T_{agg} and T_{size} reflect thermally-induced aggregation whereas B_{22} and k_D capture aggregation driven by intermolecular forces. Together, these results show that NaCl and arginine reduce thermal aggregation but promote attractive intermolecular forces and PS80 has the opposite effect.

Sucrose and NaCl decreased T_m and T_{onset} for all the tested proteins. While both are used to adjust tonicity, sucrose is also widely employed as a protein stabilizer; however, at excessive concentrations, sucrose can destabilize proteins, increasing viscosity and promoting aggregation.[5,6] It is typically used at 50 mg/mL in liquid formulations[7], approximately half the concentration used in this study, which may explain the observed destabilization. Trastuzumab's commercial formulation contains 19.09 mg/mL trehalose, another disaccharide, and the highest sugar concentration among the reference formulations, further illustrating the importance of optimizing sugar concentration. It's also possible that apparent sucrose effects reflect two-factor interactions, which can be confounded as main effects in the resolution 3 fractional-factorial design employed here.

Aunty's speed, throughput, and plate-based format make it the perfect partner for DoE, AI-based, or Bayesian optimization of formulation thermal stability. Aunty data can readily integrate with wider multi-objective formulation studies or be used to conduct higher resolution studies to refine excipient concentrations. These methods work for construct evaluation, protein engineering, or antibody conjugate screening. Together, Aunty provides the data you need to stabilize your samples.

Freeze-thaw stability

Repeated freeze-thaw cycles are a common damaging stress for biologics that can come about during the manufacturing process or because of improper storage and handling. Aggregation is the most common degradation pathway from freeze-thaw, but structural changes which impact unfolding can also occur.[8] Aunty can be used to assess these effects on protein formulations using DLS and SLS to look at aggregate formation and nanoDSF to examine protein unfolding.

After 3 freeze-thaw cycles, all proteins had higher T_ms in pH 7 than pH 5.5, indicating greater stability at neutral pH (Table 4). NaCl and sucrose decreased the T_ms across proteins, while arginine increased the T_m of the trastuzumab biosimilar and carbonic anhydrase. Arginine and NaCl increased T_{agg} and T_{size}, whereas PS80 decreased them. Therefore, arginine and NaCl reduce thermal aggregation while PS80 makes it more likely. No significant aggregation was detected by DLS following freeze-thaw (data not shown). These results are consistent with the earlier excipient screen.

Protein	T_m/T_{onset}		T_{agg}/T_{size}	
	+	-	+	-
Adalimumab	pH 7	NaCl	Arginine NaCl	PS80
Rituximab	pH 7	NaCl Sucrose PS80	Arginine NaCl	PS80
Trastuzumab	pH 7 Arginine	Sucrose NaCl	Arginine NaCl	PS80
Carbonic anyhydrase	Arginine pH 7	Sucrose NaCl	Arginine pH 7	Sucrose

Table 4: Excipients which significantly increased (+ columns, green) or decreased (- columns, red) the indicated stability metrics after 3 freeze-thaw cycles for each of the proteins, based on regression analysis. The order of the excipients reflects the relative magnitude of the effect.

Unlike the mAbs, carbonic anhydrase had noticeable differences in aggregation behavior after repeated freeze-thaw (Figure 5). Although T_{agg} values were high in Formulations 2 and 6, repeated freeze-thaws significantly decreased them, from 62.8 to 56.4 °C and 66.7 to 61.5 °C, respectively. Arginine is the only common component between formulations 2 and 6, suggesting it acted as a cryosensitizer for carbonic anhydrase despite reducing aggregation more broadly. The inclusion of NaCl and PS80 mitigated this effect in formulations 4 and 8.

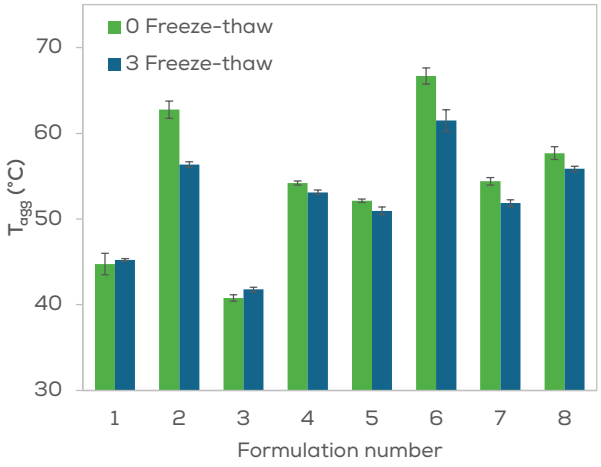

Figure 5: T_{aggs} of carbonic anhydrase in 8 formulations before (green) and after (blue) 3 freeze-thaw cycles. Data is shown as the average of triplicate measurements; error bars are 1 standard deviation.

Comparing the SLS curves of Formulations 2, 4, and 6 more clearly illustrates the complex impact of freeze-thaw cycles on carbonic anhydrase (Figure 6). Formulation 4 exhibited few changes in its SLS profile before and after 3 freeze-thaws, but the SLS intensity of Formulations 2 and 6 increased earlier in previously frozen samples, consistent with earlier aggregation onset. Formulation 6 also reached a substantially higher SLS intensity at elevated temperatures in the 3x freeze-thawed sample, suggesting formation of either larger or more numerous aggregates. All three Formulations displayed a sharp loss of SLS signal above 65 °C in the 3x freeze-thawed sample, which can occur when a protein precipitates out of solution. Overall, carbonic anhydrase in Formulation 2 and 6 aggregated more readily and more severely under thermal stress following repeated freeze-thaw cycles.

Aunty's multi-parameter measurements made clear identification of factors driving instability after freeze-thaw cycles simple and helped find ways to mitigate the effects using excipients and optimal pH. By capturing conformational stability and aggregation behavior under thermal stress, these experiments provide actionable insight into degradation pathways, supporting more informed formulation design and downstream risk assessments.

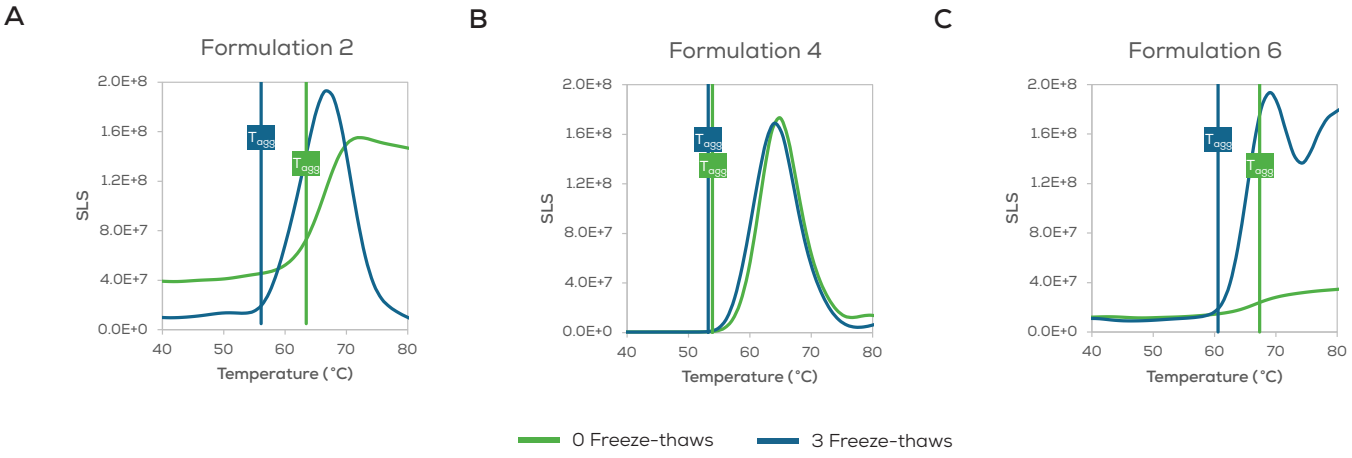

Figure 6: SLS intensity versus temperature of carbonic anhydrase in Formulations 2 (**A**), 4 (**B**), and 6 (**C**) before (green) and after (blue) 3 freeze-thaw cycles. T_{aggs} are indicated by vertical droplines. Curves are representative of triplicate measurements.

Conclusion

DoE studies, Bayesian models, and AI tools help make sense of the increasingly complex world of protein and mAb formulations, but only if they're supported by high-throughput, multi-parameter stability data. Aunty is up to the challenge of delivering this data with its one-of-a-kind combination of plate-based assays for conformational and colloidal stability in a single platform. The result is faster, data-rich screening with minimal sample use, enabling quicker, more confident formulation decisions to move therapies forward, from the lab to the clinic and into patients that need them.

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Unchained Labs
4747 Willow Road
Pleasanton, CA 94588
Phone: 1.925.587.9800
Toll-free: 1.800.815.6384
Email: info@unchainedlabs.com

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