NANOTEMPER

APPLICATION NOTE

Stability optimization of engineered mAbs

Fahimeh Raoufi[1], Marc Bailly[1], Laurence Fayadat-Dilman[1], Brett Thurlow[2], Stefanie Kall[2]

[1]Merck & Co, Discovery Biologics, Protein Sciences, South San Francisco, CA USA

[2]NanoTemper Technologies , Germany

Abstract

During biologics development, it is critical to ensure stability of a monoclonal antibody (mAb) with the ultimate goal of reaching the clinic. Biologics discovery often involves huge libraries of candidates with varying biophysical characteristics, which need to be evaluated and optimized for greater developability and downstream success. Understanding how candidate sequence attributes alter biophysical parameters is necessary for improved rational design and delivery of biological candidates. Examining how specific mutations alter the biophysical profile of a mAb is an important first step in the candidate selection and developability workflow. **Here, the Protein Sciences Department within Biologics Discovery at Merck used the Prometheus Panta and the parameters obtained from nano-differential scanning fluorimetry (nanoDSF), backreflection (turbidity), and dynamic light scattering (DLS) to characterize a selection of monoclonal antibodies with sequence diversity.**

Introduction

Researchers involved in the discovery phase of therapeutic biologics, particularly monoclonal antibodies, require many parameters to assess the characteristics of their candidates to ensure their streamlined development and long-term success in the clinic. Early phase discovery involves vast libraries of candidates with sequence and epitope diversity. Establishing a better understanding of how mutations in the sequence of the antibodies change biophysical parameters of these diverse candidates helps drive sequence selection.

With Prometheus Panta, it is now possible to do dynamic light scattering (DLS) experiments in tandem with nano-differential scanning fluorimetry (nanoDSF) and backreflection along an entire thermal ramp in order to gain conformational, thermal stability, and turbidity information about your formulation[1]. Prometheus's nanoDSF and turbidity measurements obtained from the NT.Plex instrument have been validated as industry-standard, but with the addition of DLS capabilities, it is crucial to ensure the same quality and reliability can be found in the Panta instrumentation.

Merck's Protein Sciences group recently used the Prometheus Panta to evaluate the properties of a series of monoclonal antibody mutants with the objective of supplying data to machine learning programs to improve the candidate selection process[2]. **DLS, nanoDSF, and backreflection were used to evaluate sequence mutations and determine how they affected molecule stability**.

It was crucial not only to evaluate these candidates with the added DLS ability, but also to determine whether the Panta could be integrated into their workflow without a loss in measurement accuracy from the nanoDSF and turbidity measurements they are typically performing. **Once the data were collected in the Panta, they compared data for the same molecules measured previously on the Prometheus NT.Plex to ensure repodicibility.**

Methods

Prometheus Panta measurements

Antibody samples were purified to 1 mg/ml in 20 mM sodium acetate pH 5.5 buffer. Single replicates of each sample were run in the Early Access Program (EAP) Prometheus Panta with the 48 individual capillary adapter in place. UV LED excitation was 12% for nanoDSF and turbidity measurements; excitation was set to 100% for DLS experiments. Isothermal measurements for DLS were collected at 25°C using the high-sensitivity mode prior to the thermal denaturation experiments. Temperature denaturation with nanoDSF, backreflection, and DLS acquisition was run from 25-95°C at 1°C/min. Data was collected using a beta version of Panta.Control software, and after converted to a format to allow analysis using Panta.Analysis software v1.1.

Prometheus NT.Plex measurements

Single replicates of each sample were run in the Prometheus NT.Plex. UV LED excitation was 40% for nanoDSF and turbidity measurements. Temperature denaturation with nanoDSF and backreflection acquisition was run from 25-95°C at 1°C/min. Data was collected using version 2.2 of Therm.Control software.

©2021 NanoTemper Technologies, Inc. South San Francisco, CA, USA. All Rights Reserved.

Results

Evaluation of two unique families of mAbs

IgG-based mAb candidates had mutations introduced in their non-binding regions to profile how single- and double- site mutations altered the stability profiles of these molecules. The aim was to better understand how the genetic and therefore proteomic diversity of early phase antibodies were affected by small changes in the structural core. Each antibody and its mutants were evaluated using nanoDSF, turbidity, and DLS along a thermal ramp to characterize their biophysical characteristics and determine how the mutations affected their stability.

When examining the thermal denaturation profiles of the mAbs, it is easy to see their varying behavior. In Figure 1A, four antibodies "Group 1" exhibit different melting temperatures (T_m) via nanoDSF. Additionally, two antibodies (Protein A and Protein D) exhibit significant turbidity accumulation, while Proteins B and C only have modest turbidity signal increase; this is reflected in the cumulant radius data, which also shows an increased cumulant radius at the onset of the experiment for Protein D.

For Group 2, the six antibodies show pronounced differences in their nanoDSF unfolding profiles in Figure 1B. Likewise, there are significant differences in their turbidity and cumulant radii changes. Notably, only Protein G shows no major turbidity changes, which is also reflected in its cumulant radius. Detailed parameter measurements can be found in Table 1.

Taken together, these results demonstrate the value of simultaneous measurements of DSF, nanoDSF, and backreflection along a thermal gradient. The results demonstrate the structural variation among IgG-derived mAbs, even within related groups. The values for the antibodies can be quantified to evaluate their fitness for development, and to determine how mutations in the genetic sequence can alter biophysical parameters.

Nomenclature

- **T_{m1}** First melting inflection temperature, at which 50% of the least thermalstable domain is unfolded
- **T_{on}** Onset of unfolding, temperature at which deviation from linear baseline exceeds 0.5%
- **T_{agg}** Onset of aggregation, temperature at which scattering deviates >1.1x of initial value (also T_{size}, $T_{scattering}$)
- **T_{turb}** Onset of turbidity, temperature at which backreflection signal deviates from linear baseline by > 0.5% (Referred to as T_{agg} in Prometheus PR.Plex instruments)
- **PDI** Polydispersity Index, a measure of the heterogeneity of particles in a sample; lower values indicate a more homogenous mixture

©2021 NanoTemper Technologies, Inc. South San Francisco, CA, USA. All Rights Reserved.

	T_{m1}	T_{on}	T_{turb}	T_{agg}	PDI (25°C)
Protein A	64.99	60.48	64.33	63.42	0.1
Protein B	74.42	65.23	83.78	75.71	0.03
Protein C	70.13	63.47	78.48	79.16	0.01
Protein D	70.43	63.47	82.91	80.84	1.14
Protein E	63.91	54.65	59.95	60.41	0.33
Protein F	69.06	57.30	79.39	68.28	1.17
Protein G	71.47	65.26	82.97	82.34	0.03
Protein H	69.70	63.52	69.68	65.47	0.01
Protein I	69.69	62.61	76.77	70.71	0.04
Protein J	69.69	62.62	67.93	67.10	0.01

Table 2: Summary data of first unfolding temperature, unfolding onset, turbidity onset, scattering onset, and PDI for each parent in two groups. A PDI <0.1 is considered highly monodisperse; anything above 0.25 is considered highly polydisperse -- high PDI values highlighted in red.

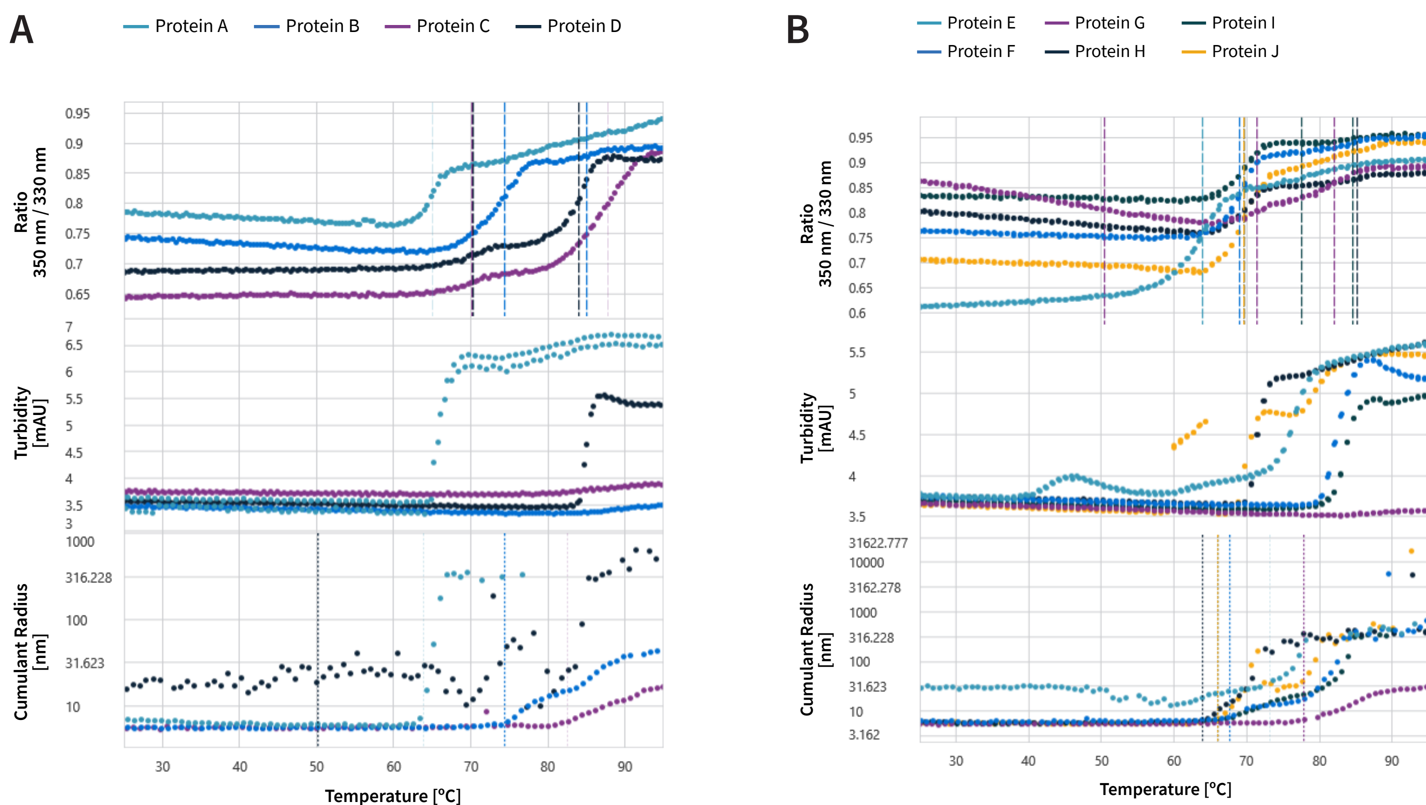

Figure 1: Profiles of parents from Group 1 (A) and Group 2 (B) antibody groups have unique thermal profiles from intrinsic nanoDSF (top), turbidity (center), and DLS cumulant radius measurement (bottom).

©2021 NanoTemper Technologies, Inc. South San Francisco, CA, USA. All Rights Reserved.

Evaluation of mutations on antibodies to determine how they alter multiple stability parameters

With the establishment of initial T_m, T_{on}, r_H, and PDI values for the non-mutated antibodies, it is possible to determine whether the mutant mAbs have significant deviations even with only modest structural alterations.

Here we have selected two antibody groups and their mutants, representative from each group of mAbs. In Figure 2A, we see Protein A and its mutants A1-4. Note that overall, the changes the mutations made to profiles was minimal compared to the differences in the initial groups, as seen in Figure 1A and 1B. In Figure 2B, we see similar results for Protein G, where the nanoDSF and turbidity data have broadly similar shapes to the non-mutated molecule; note that the non-mutated molecule's cumulant radius does not indicate a large change in size until a much higher temperature than the mutant molecules.

When examining Table 2, it becomes evident that the mutant mAbs did not exhibit significantly increased stability, either thermal or conformational, compared to the non-mutated. However, it also exemplifies the differences that structural changes from single or double mutations can introduce to a molecule. **Small changes in the protein sequence can have significant and measurable effects on the biophysical characteristics of a mAb.**

©2021 NanoTemper Technologies, Inc. South San Francisco, CA, USA. All Rights Reserved.

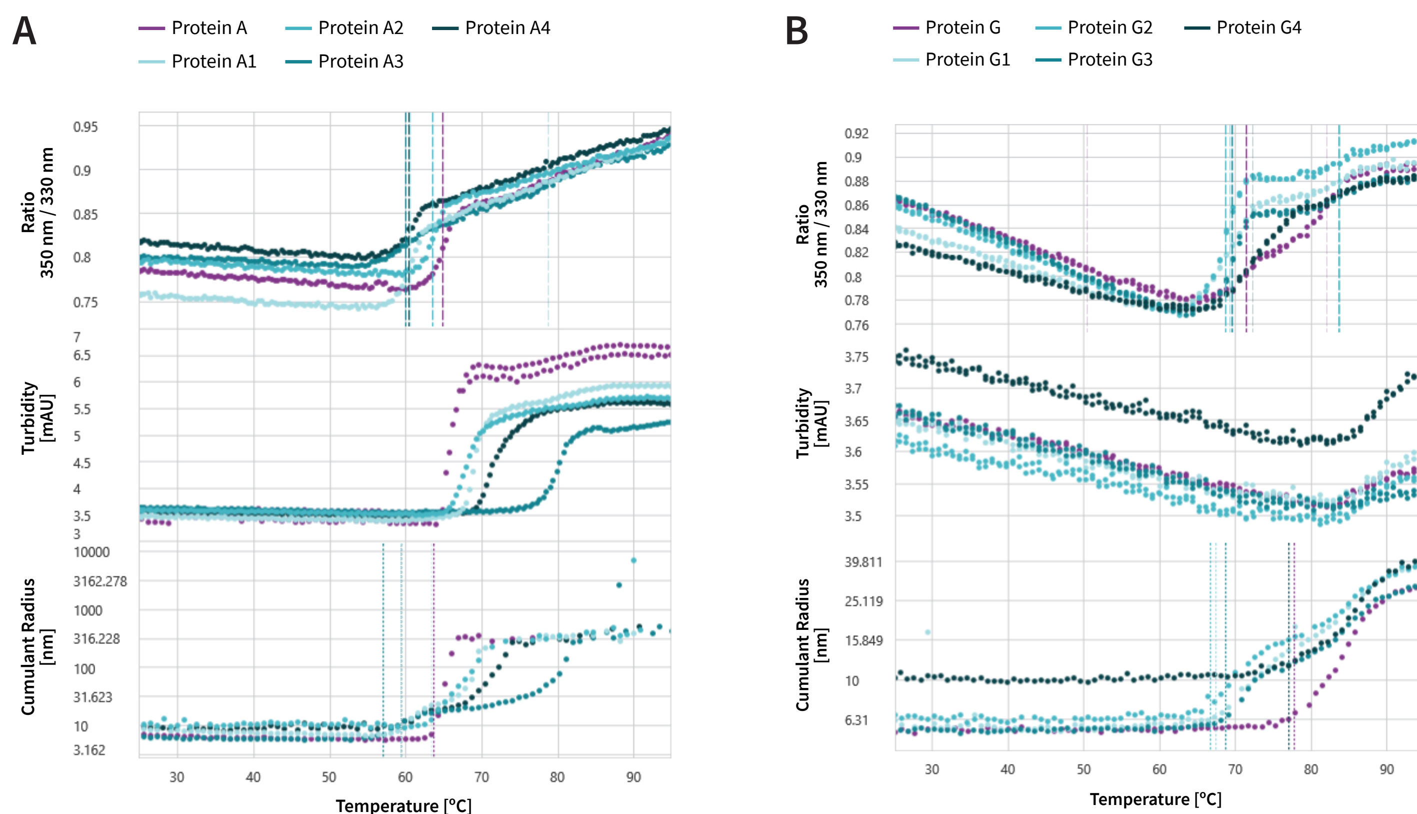

Figure 2: Profiles of two "groups" within each group structure. A is Protein A (non-mutated) + Protein A1-4 (mutants). B is Protein G (non-mutated) + Protein G1-4 (mutants). Each mutant represents only 1-2 site mutations in a non-antigen-binding region of the IgG-derived mAb. Note the overall profile shapes remain similar for DSF (top), turbidity (centre), and DLS (bottom), though the mutations have a noticeable effect on the structural profile.

	T_{m1}	T_{on}	T_{turb}	$T_{scattering}$	PDI (25°C)
Protein A	64.99	60.48	64.33	63.42	0.1
Protein A1	60.69	56.93	60.47	60.72	0.08
Protein A2	63.69	59.57	64.86	63.38	0.77
Protein A3	60.08	54.91	71.91	58.96	0.02
Protein A4	60.63	56.40	61.29	60.71	1.23
Protein G	71.47	65.26	82.97	82.34	0.03
Protein G1	69.31	54.70	83.81	69.07	0.04
Protein G2	68.76	59.99	83.81	69.05	0.30
Protein G3	69.69	63.52	81.20	69.04	0.02
Protein G4	72.27	63.52	83.81	72.55	0.64

Table 2: Summary data of first unfolding temperature, unfolding onset, turbidity onset, scattering onset, and PDI for each group member for two representative groups. Non-mutated in **bold**. A PDI <0.1 is considered highly monodisperse; anything above 0.25 is considered highly polydisperse -- high PDI values highlighted in red.

©2021 NanoTemper Technologies, Inc. South San Francisco, CA, USA. All Rights Reserved.

Evaluating and comparing data from Promethus Panta vs NT.Plex

One of the most important tenets of research is independent reproducibility. At large pharma companies such as Merck, stability parameters may be tested by different individuals across multiple sites, and it is crucial that these results can be independently verified.

With the recent integration of DLS measurements in the Prometheus line, it was necessary to evaluate the reliability of the nanoDSF and turbidity data obtained from different systems. Previously published and evaluated data from Prometheus NT.Plex instrumentation must be replicated on the Panta to ensure it can be fully integrated into already established protocols.

The mAbs in this study were previously evaluated using the Prometheus NT.Plex, with the same experimental parameters for nanoDSF and turbidity measurements, but by a different researcher. Figure 3 shows a few representative corroborations between the individual data points collected on the Panta vs. the NT.Plex. **There is high correlation for the candidates evaluated, demonstrating good agreement between the new instrumentation and the established technology in the field.**

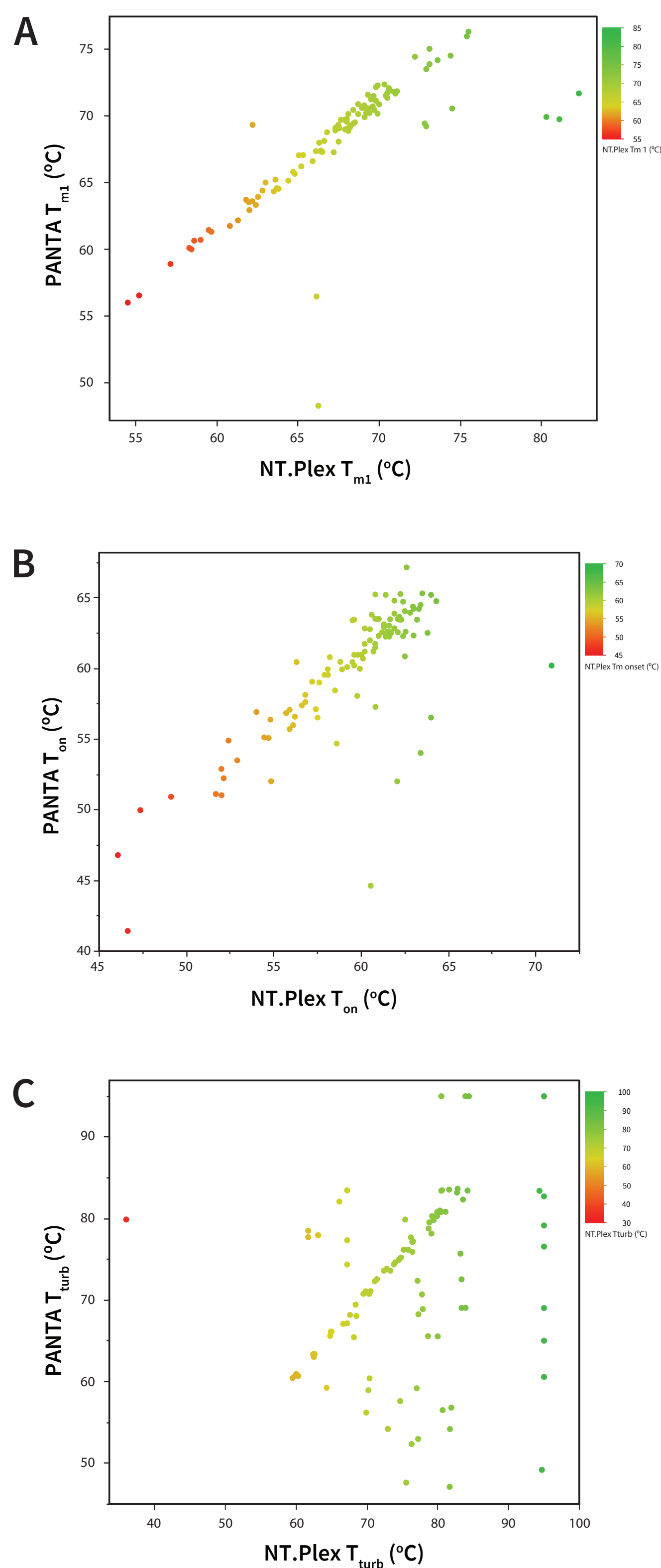

Figure 3: Figure 3 shows the correlations between onset of melting (A), first melting temperature inflection point (B), and onset of turbidity (C, particles >25 nm diameter) correlate well between the Panta and the NT.Plex.

©2021 NanoTemper Technologies, Inc. South San Francisco, CA, USA. All Rights Reserved.

One of the benefits of adding DLS to the evaluation matrix becomes evident when evaluating T_{on} and T_m. In Figure 4A, we show that these parameters are highly, though not perfectly correlated. While characterizing antibodies, when these discrepancies arise, it is important to have additional parameters for evaluation. With DLS, the $T_{scattering}$ and PDI information help resolve cases where the T_m and T_{on} do not fall within the expected range. Often these cases indicate unfolded or destabilized species prior to the experiment (via high PDI) or can help validate the rate of unfolding (by monitoring the $T_{scattering}$). Likewise, close examination of the size distribution can reveal biophysical changes occurring between the onset and inflection of melting.

Figure 5 shows the high correlation between T_{m1}, T_{on}, T_{turb} (formerly T_{agg}), and turbidity inflection point (IP) measured by both the Panta and the NT.Plex. It also shows strong correlation with the scattering IP and cumulant radius change IP from DLS data and the thermal unfolding, as measured by the NT.Plex. Notably, measurements related to the DLS parameters of hydrodynamic radius (r_H) and PDI do not have strong correlation to thermal stability behavior. DLS parameters can be used to supplement thermal unfolding-based data to have additional ranking parameters for antibodies.

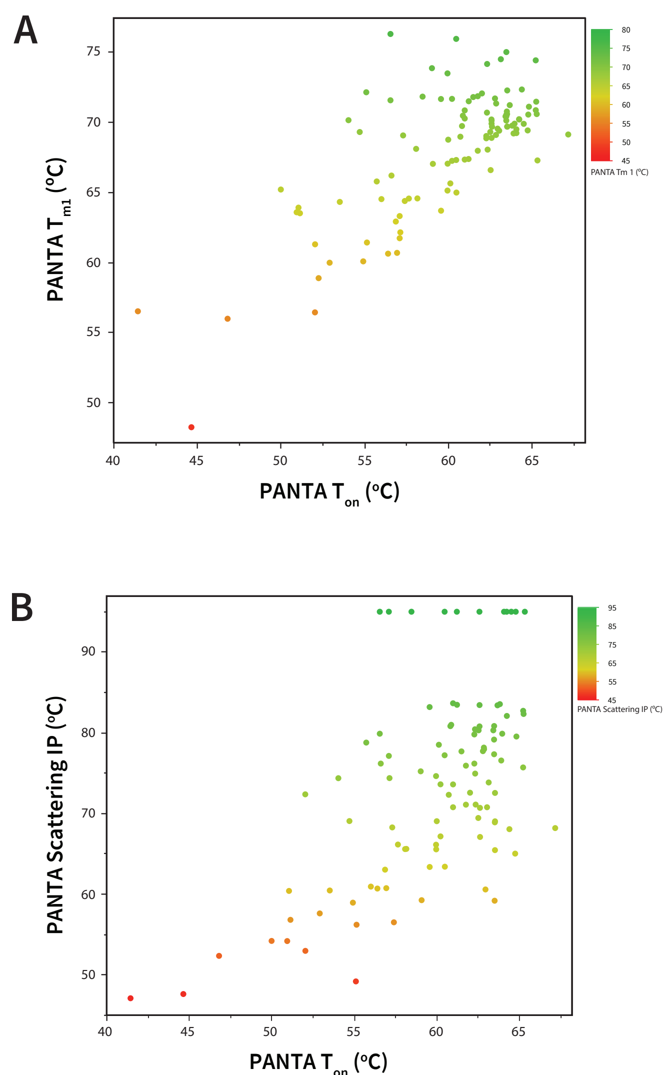

Figure 4: Figure 4a demonstrates that while first melting temperature and unfolding onset temperature are correlated, they do not line up perfectly. In 4B, with the additional parameter of scattering IP, we gain deeper insight to the unfolding behavior. Note the antibodies with the higher melting onsets (dark green) cluster along the right side of the graph and can be further refined for beneficial properties using the scattering IP.

©2021 NanoTemper Technologies, Inc. South San Francisco, CA, USA. All Rights Reserved.

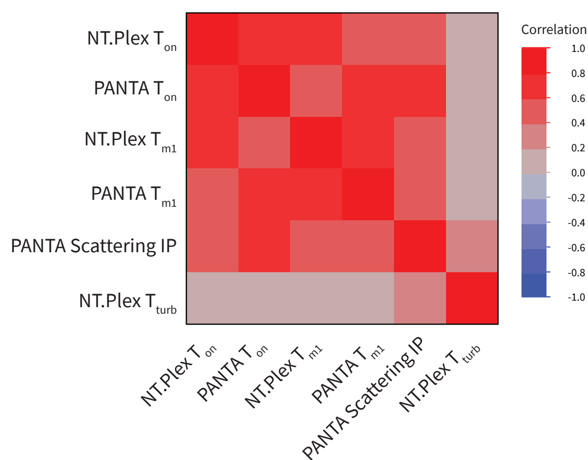

Figure 5: Clustering of correlations from data obtained from the Prometheus Panta vs Prometheus NT.Plex as plots showing individual data points (top) and heat maps (bottom). There is a strong correlation between parameters obtained on the two different instrument types for T_m, T_{on}, Turbidity inflection point (IP), and T_{turb}. DLS parameters PDI and r_H do not exhibit strong correlation to the thermal stability data (data not shown).

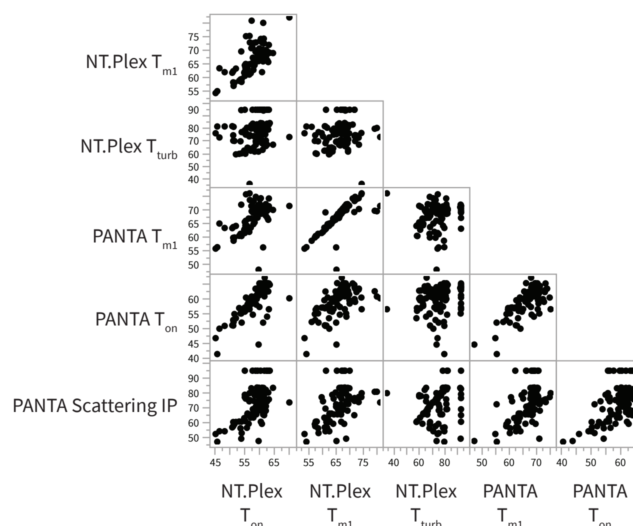

Figure 6: Figure 5 shows the high correlation between T_{m1}, T_{on}, T_{turb} (formerly T_{agg}), and turbidity IP measured by both the Panta and the NT.Plex. It also shows strong correlation with the scattering IP and cumulant radius change IP from DLS data and the thermal unfolding, as measured by the NT.Plex. Notably, measurements related to the DLS parameters of hydrodynamic radius (r_H) and PDI do not have strong correlation to thermal stability behavior. DLS parameters can be used to supplement thermal unfolding-based data to have additional ranking parameters for antibodies.

Conclusions

There is a complicated matrix of biophysical parameters to evaluate when determining how mutations affect an antibody's stability. Thermal and conformational stability reflect only part of the picture of what characterizes stability parameters. Biologics researchers evaluate a wealth of information to better understand their molecules' early developability profile, and no single experiment tells them everything they need regarding a molecule's attributes.

Though there are often correlations between different stability parameters, they are not strictly linked; for example, a mAb with high thermal stability as reflected by high T_m and T_{on} may also demonstrate a high PDI, therefore

©2021 NanoTemper Technologies, Inc. South San Francisco, CA, USA. All Rights Reserved.

demonstrating unfavorable biophysical characteristics. Other important biological properties, such as the pI of the protein or its hydrophobicity index, have impact downstream when it comes to packaging of the material or delivery of the therapeutic to the patient. Classifying a library with extensive genetic variation is crucial for understanding early discovery candidates and how to ultimately optimize them for long-term success in the clinic.

For evaluation of some basic biophysical characteristics, Prometheus Panta can give you important parameters such as T_{on},T_m, T_{agg}, and PDI, which can not only give a biophysical picture of antibody libraries, but also predict the long-term stability of a given antibody. **Here we have demonstrated the value of these parameters for ranking candidates, as well as that the nanoDSF and turbidity-derived data from the Panta corroborates well with previously obtained data from the Prometheus NT.Plex**. With this information, we can conclude that the Panta instrumentation can be integrated into workflows with groups that have already collected data on the NT.Plex.

©2021 NanoTemper Technologies, Inc. South San Francisco, CA, USA. All Rights Reserved.

References

1 P. Schramm, et al. Sizing accuracy and intra-assay precision of DLS measurements with Prometheus Panta. NanoTemper Technologies Tech Note. 2021.

2 M.A. Bailly, et al. Predicting Antibody Developability Profiles Through Early Stage Discovery Screening. MAbs. Jan-Dec 2020;12(1):1743053. doi: 10.1080/19420862.2020.1743053

NANOTEMPER

Prometheus is a trademark of NanoTemper Technologies GmbH, Munich, Germany. NanoTemper is a registered trademark of NanoTemper Technologies GmbH, Munich, Germany. ©2021 NanoTemper Technologies, Inc. South San Francisco, CA, USA. All Rights Reserved.