

# Screening Constructs with $\Delta G$ and the Hunk

## Introduction

Automating isothermal chemical denaturation experiments with Hunk enables you to measure the Gibbs free energy ( $\Delta G$ ) of biologics easily and with little hands-on time.  $\Delta G$  is a quantitative stability measurement that allows for the determination of the ratio of folded to unfolded protein at ambient temperatures for samples at equilibrium (Reference 1). Quantifying stability with  $\Delta G$  measurements can help researchers overcome the challenge of ranking constructs or formulations by qualitative measurements such as thermal ramp stability ( $T_m$  or  $T_{onset}$ ). By pairing  $T_m$  and  $\Delta G$  stability measurements, the best candidates can be identified with greater confidence.

This application note describes how  $\Delta G$  can be used as an orthogonal approach to traditional thermal ramp stability measurements like  $T_m$ , to help identify the most promising monoclonal antibody (mAb) construct candidates (Figure 1). The stability of ten mAb constructs was screened and qualitatively ranked with  $T_m$  measurements. A follow-up  $\Delta G$  screen was then used to refine the  $T_m$  stability ranking to determine the top two candidates.

Hunk fully automates the measurement of  $\Delta G$ , making chemical denaturation much less laborious. Hunk formulates chemical denaturation curves from supplied stock solutions, incubates samples, reads the fluorescence emission spectra and analyzes the resulting chemical denaturation curves.  $\Delta G$  measurements quantify stability at ambient temperatures and can be used to rank and refine stability in construct screens, formulation screens and optimization experiments.

## Methods

Ten mAb constructs were prepared in PBS pH 7.4 at 325  $\mu\text{g}/\text{mL}$  and 1  $\text{mg}/\text{mL}$ . Thermal ramp experiments were performed on Uncle to measure  $T_m$ . Three Unis were loaded, in triplicate, with 9  $\mu\text{L}$  of

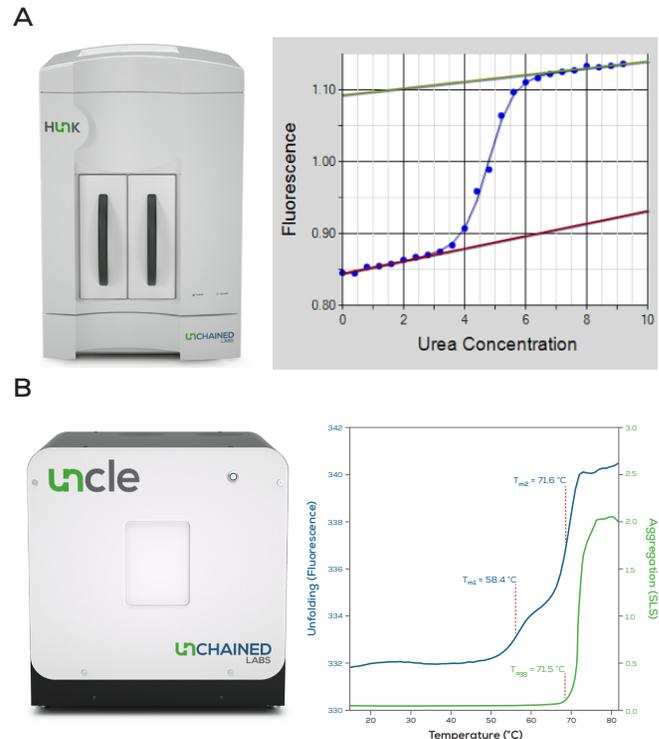


Figure 1: A: Hunk automates chemical denaturation experiments by preparing the formulations and denaturation points, and reading the fluorescence.  $\Delta G$  measurements provide quantified equilibrium stability at ambient temperatures, and can be used to refine the stability rankings based on  $T_m$ . B: Uncle provides 11 different stability applications using fluorescence, static light scattering (SLS) and dynamic light scattering (DLS).  $T_m$  values can be measured quickly with the thermal ramp application for qualitative stability ranking.

each 1  $\text{mg}/\text{mL}$  sample. A thermal ramp from 20–95 °C was run with a 0.25 °C/minute ramp rate.  $T_m$  was calculated using the barycentric mean (BCM) of fluorescence emission in the Uncle Analysis software.

Isothermal chemical denaturation experiments were performed on Hunk to measure  $\Delta G$ . Twenty-four point chemical denaturation curves were collected on Hunk using 10 M Urea as the denaturant. Hunk prepared each point in triplicate by mixing PBS pH 7.4, PBS pH 7.4 with 10 M Urea, and 325  $\mu\text{g}/\text{mL}$  of each construct to create a linear Urea gradient with 26  $\mu\text{g}/\text{mL}$  of protein in each well. Samples were incubated at 25 °C for 3 hours prior to data collection. As Hunk synchronizes

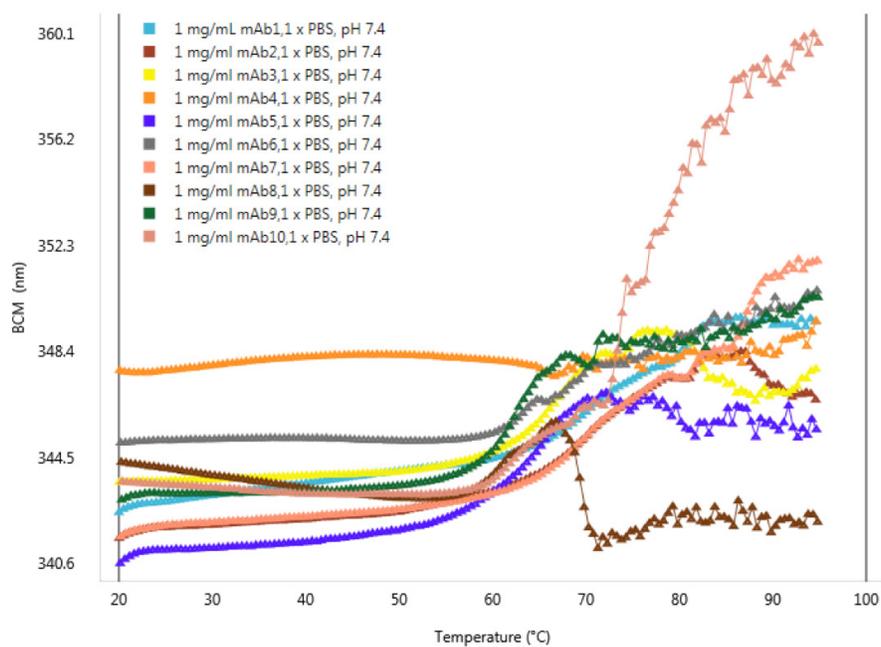


Figure 2: A thermal ramp experiment was performed for the ten mAb constructs on Uncle.  $T_m$  was determined for each unfolding transition and values were used to create a tiered ranking of construct stability.

sample preparation and data collection, traditional overnight incubation periods are not necessary.  $\Delta G$  was calculated in the Hunk software by fitting a 2-state transition in fluorescence emission intensity for each denaturation curve.

## Results

A thermal ramp experiment was performed on Uncle to measure the  $T_m$  for each construct. A wide spectrum of unfolding behavior was observed, ranging from one construct that did not unfold, to a construct that underwent 3 distinct unfolding transitions over the temperature ramp (Figure 2). This experiment was completed very quickly and required very little purified protein, which makes it an excellent primary screening method. However,  $T_m$  is a qualitative stability measurement, which makes it difficult to distinguish stabilities when temperatures are similar. Constructs were placed into one of three tiers to rank stability (Table 1). Tier 1 constructs, mAb1, mAb2, and mAb7, had  $T_{m1} > 69$  °C and  $T_{m2} > 80$  °C. mAb4 did not undergo any unfolding transitions during the thermal ramp, indicating a high level of thermal stability and was included in Tier 1. mAb3 and mAb5 underwent

unfolding transitions at lower temperatures, with  $T_{m1}$  values in the mid-60's °C and  $T_{m2}$  values in the mid-70's °C. The four least thermally stable mAbs (mAb6, mAb8, mAb9 and mAb10) had  $T_{m1} < 64$  °C and  $T_{m2} < 74$  °C. Measuring  $T_m$  provided a quick means to qualitatively rank constructs, but left

Construct	$T_{m1}$ (°C)	$T_{m2}$ (°C)	$T_{m3}$ (°C)	Tier
mAb1	70.1	80.5	n.d.	1
mAb2	69.8	81.6	n.d.	1
mAb4	n.d.	n.d.	n.d.	1
mAb7	69.4	81.7	86.4	1
mAb3	67.2	74.7	n.d.	2
mAb5	66.0	n.d.	n.d.	2
mAb6	62.9	68.9	n.d.	3
mAb8	60.3	63.7	n.d.	3
mAb9	62.6	71.5	n.d.	3
mAb10	62.4	73.6	n.d.	3

Table 1:  $T_{m1}$ ,  $T_{m2}$ , and  $T_{m3}$  were calculated from the thermal ramp experiment performed in Figure 3. Three stability tiers were determined with Tier 1  $T_{m1} > 69$  °C, Tier 2  $69$  °C  $> T_{m1} > 66$  °C and Tier 3  $T_{m1} < 66$  °C.

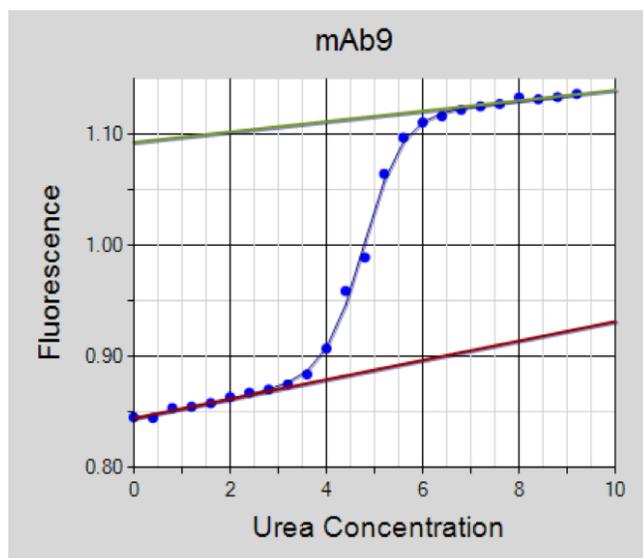


Figure 3: The Urea denaturation curve for each construct was fit by selecting the native state baseline (red) and denaturated state baseline (green) to calculate the native state  $\Delta G$  for each construct. The parameters  $C_{1/2}$ , the denaturant concentration at the transition midpoint, and  $m$ , rate of transition of  $\Delta G$  between the native and denatured state, were obtained from the nonlinear least squares fit of the denaturation curve and used to calculate  $\Delta G$  with the equation  $\Delta G = C_{1/2} \cdot m$ .

several viable candidates. To further refine construct stability rankings, a  $\Delta G$  screen was run on Hunk.

Hunk was used to quantify construct stability by collecting and analyzing 24-point Urea denaturation curves. The  $\Delta G$  for each construct was calculated from the non-linear least squares fits of the denaturation curves (Figure 3). All constructs underwent a single unfolding transition with  $\Delta G$  values ranging from 5.3–8.8 kcal/mol (Figure 4). The determination of  $\Delta G$  allowed for the refinement of stability rankings by providing stability information orthogonal to  $T_m$ .

The top two constructs, mAb1 and mAb4, were quickly identified when assessing  $T_m$  and  $\Delta G$  in tandem (Table 2). Thermal ramp experiments did not yield a  $T_m$  for mAb4, but a high degree of thermal stability was indicated by the absence of an unfolding transitions.  $\Delta G$  confirms the stability of mAb4 with  $\Delta G = 8.8$  kcal/mol. The second highest  $\Delta G$  was measured for mAb1, which was classified as a Tier 1 construct based on  $T_m$ . Pairing  $T_m$  with  $\Delta G$  reduced the top candidates from four to two, with high confidence in both. Conversely the two

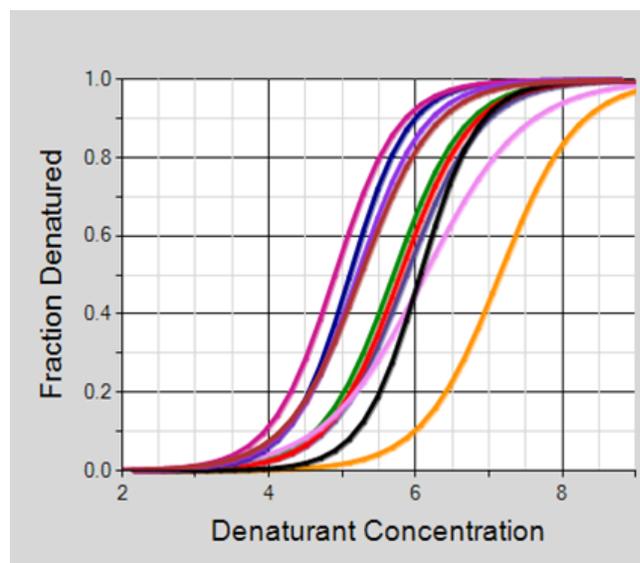


Figure 4: The overlay of the denaturation curve from 2 M to 9 M Urea for mAb1 (orange), mAb2 (blue), mAb3 (pink), mAb4 (black), mAb5 (red), mAb6 (dark blue), mAb7 (green), mAb8 (brown), mAb9 (magenta) and mAb10 (purple) displays the differences in chemical denaturation profiles.  $C_{1/2}$  varies from 4.5 M to 6 M Urea and the rate of transition between the native and denatured state varies from steep to gradual.

Construct	Average $\Delta G$ ( $\pm 0.3$ kcal/mol)	$\Delta G$ Rank	$T_m$ Tier
mAb4	8.8	1	Tier 1
mAb1	8.1	2	Tier 1
mAb6	7.7	3	Tier 3
mAb5	7.3	4	Tier 2
mAb9	7.0	5	Tier 3
mAb2	6.9	5	Tier 1
mAb7	6.9	5	Tier 1
mAb10	6.9	5	Tier 3
mAb8	6.6	9	Tier 3
mAb3	5.3	10	Tier 2

Table 2:  $\Delta G$  was used to refine construct stability ranking.

least stable constructs as determined by  $\Delta G$  are mAb3 and mAb8, which are in Tier 2 and Tier 3 for  $T_m$ , respectively. These two constructs can be eliminated from further consideration.

$\Delta G$  also provides valuable information when assessing middle-of-the-pack constructs. For example, mAb6 was ranked in Tier 3, but has a  $\Delta G = 7.7$  kcal/mol, while the Tier 1 constructs mAb2 and mAb7 have a  $\Delta G = 6.9$  kcal/mol. The three mAbs are considered relatively stable, but the difference in  $\Delta G$  corresponds to having 2.9 ppm or 11.4 ppm of unfolded protein in the native state for mAb6 and mAb2/mAb7 respectively. Additionally,  $\Delta G$  is measured at ambient temperatures and does not require thermal stress like  $T_m$  measurements. Therefore, picking additional candidates or back-up molecules based on  $\Delta G$  may prevent bias in the selection of constructs towards thermal stability. This has the additional benefit of increasing construct diversity prior to beginning optimization or formulation screening.

## Conclusion

Measuring  $\Delta G$  with Hunk during construct screening provided additional insight into the stability of the ten mAbs studied and helped to fill in the gaps left by  $T_m$ . Hunk narrowed the list of top construct candidates to two. The stability of mAb1, a top candidate based on  $T_m$ , was confirmed and a  $\Delta G$  value quantified the stability of the thermally stable construct, mAb4, which did not yield a  $T_m$  value. Including  $\Delta G$  in the analysis of constructs also adds an orthogonal measurement of stability at equilibrium and ambient temperatures that can help researchers identify a diverse selection of constructs for further study. Hunk gives researchers the tool to make chemical denaturation and the measurement of  $\Delta G$  accessible during construct evaluation.

## References

- 1 Freire E, Schön A, Hutchins BM, Brown RK. 2013. Chemical denaturation as a tool in the formulation optimization of biologics. *Drug Discov Today* 18(19-20): 1007–1013.



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