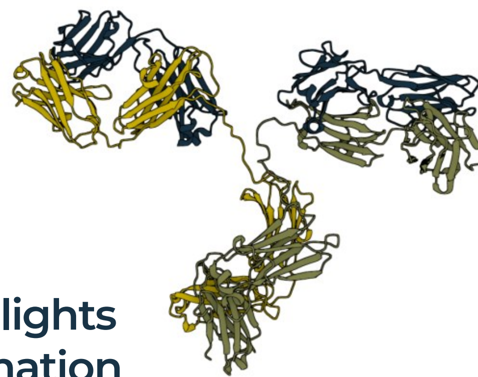
Protein Stable

Applied Innovations in Protein Characterisation



Statistical Repeatability of Tm Values Highlights Utility of Protein Stability Screening Automation

Introduction

Differential Scanning Fluorimetry (DSF) is a powerful technique for assessing protein stability, particularly concerning early-stage screening of proteins (e.g. different mutants), or later stage final product formulations to help determine shelf life and product quality. In addition, screening for ligands binding to a target protein to quickly compare functionality of a large set of chemical compounds is achievable using DSF.[1] Traditional techniques for assessing protein stability like Differential Scanning Calorimetry (DSC) can be time-consuming and further require higher amounts of sample to use. Differential Scanning Fluorimetry can be performed either using extrinsic dyes to label proteins or increasingly by using intrinsic fluorescence of the cyclic amino acids making up the protein structures. Whilst extrinsic dyes are highly sensitive, they do add risk to the method by influencing any stability profile with their method of action. The intrinsic DSF approach removes such fears of dye influence however has so far been limited by sample requirements to achieve the sensitivity required and lack of connectivity with other techniques to increase throughputs and reduce handling. The SUPR-DSF recently introduced by Protein Stable addresses these concerns by measuring protein stability in 384-well microplates, using low volumes of sample whilst measuring intrinsic fluorescence of proteins to exclude the use of extrinsic dyes. This note further explores the capabilities of the SUPR-DSF, particularly concerning connectivity to other process instrumentation that reduces operator workload, minimises manual handling risks and increases walk-away time from the screening process. Automation of the SUPR-DSF, whether modular or fully integrated allows increased capacities, more sample or condition screening and collection of data for the user to analyse.

To illustrate the accuracy, and repeatability of microplate-based screening with the SUPR-DSF, 16 x 384-well microplates were prepared with lysozyme using a modular approach with each stage of the process running independently. Full integration of the modular workstations is easily achieved should additional automation and walk-away time be desired. Thermal denaturation data from over 6000 replicates was collected and the statistics, including mean and standard deviations presented. The whole process achieved a standard deviation of only 0.14°C, across all 16 microplates while reducing the amount of sample consumed. This not only lends confidence to the use of microplates, in the screening of proteins' conformational stability, but also shows how the modern platform of the SUPR-DSF can be connected in a wider screening protocol.

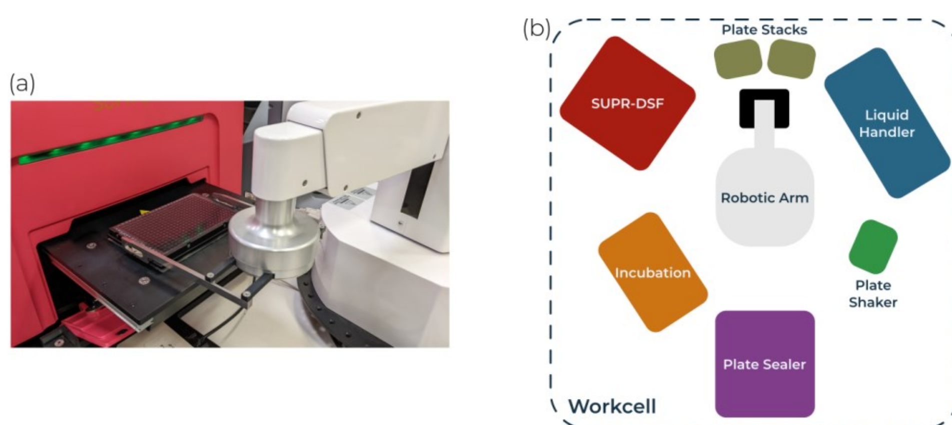


***Figure 1** - **(a)** Microplate being loaded by robotic arm. **(b)** Illustrative workcell for automatic workflow, incorporating SUPR-DSF. Stages include the plate stack for holding plates, liquid handler for dispensing samples, plate shaker to remove bubbles, plate sealer, incubation station and SUPR-DSF for measurements.*

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Table 1 *– Information about the measurement and statistics calculated from analysis of the lysozyme thermal melt curves measured across the 16 microplates.*

No. Microplates	*No. Lyso. Samples*	*T_m Mean (°C)*	*SD (°C)*
16	6096	74.45	0.14

Results

Collecting the fitted T_m values from all microplates measured gave a mean value of 74.45°C, which is consistent with values reported in the literature.[2] From **Figure 2(a)** it can be seen that the determined T_m values vary across the microplate within the standard deviation of 0.14°C (wells P22, P23 and P24 were water controls). This variation is to be expected from a biological system and therefore demonstrates the reproducibility of the SUPR-DSF. Using robotic liquid handlers for sample preparation and a centrifuge for spinning down the plates after dispensing helps improve the sample solution consistency, as was done for the plates used here. For these experiments, liquid handling and centrifugation was performed separately as process modules, though could easily be automated as a single process.

A histogram of the T_m values (No. bins = 1000) shows the expected normal distribution of values (**Figure 2(b)**) that reflect real-world measurements. Fitting a normal distribution function to the histogram data provided a standard deviation value of 0.14°C, centred around -0.02°C. The T_m values were subtracted by the mean value so the histogram would be centred at zero. Using a normal distribution function, it can be stated that 68% of the T_m values measured fall within ±0.14°C of the mean and 95% within ±0.28°C of the mean value.

Conclusion

Leveraging the microplate platform for DSF provides the advantages of lower consumable costs, lower sample usage, increased ease-of-use, and integration within existing automation technologies. However, these advantages shouldn't significantly impact the measurement performance. Analysing the statistical distribution of over 6000 repeat lysozyme samples gave accurate T_m values while providing a low standard deviation of only 0.14°C. The SUPR-DSF therefore has great utility within protein stability screening, connecting to supporting technologies, minimising handling errors and risk, offering more samples with lower sample usage.

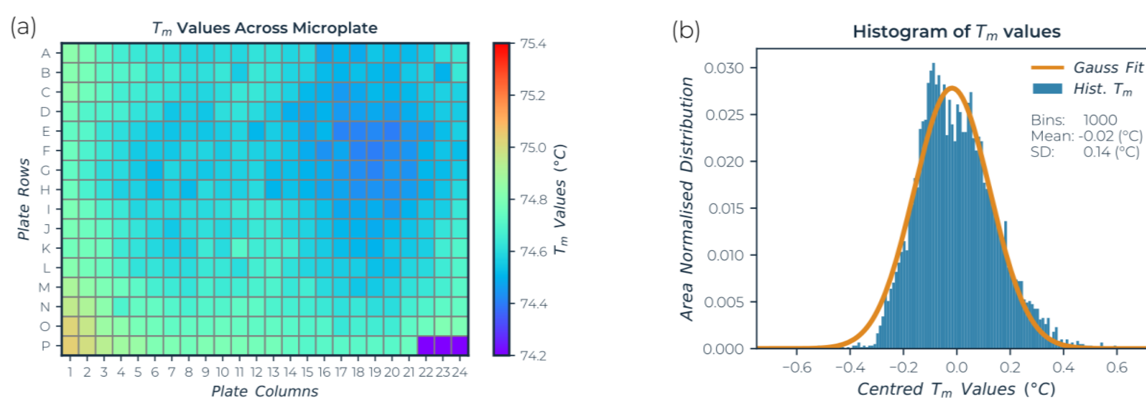

Figure 2 *– **(a)** Heatmap of the measured T_m values across on of the lysozyme plates measured. P22-P24 are water control wells. **(b)** Area normalised and centred histogram of T_m values across all 16 microplates. A normal distribution curve has been fitted to the histogram data and parameters listed within the figure box.*

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Methodology

Lysozyme Microplates

Lysozyme (Roche) from hen egg white was prepared at 0.5 mg mL^{-1} in deionised water (pH 5.7), to better align with conditions used in the literature.**[2]** The lysozyme solution was gently stirred for 10 mins to ensure the protein powder had completely dissolved without unnecessary agitation.

To prepare the 384-well PCR microplate (BioRad: #HSP3866), lysozyme stock solution was dispensed with the Dragonfly® liquid handler (SPT LabTech). The option of automated liquid handling ensured that the sample volume was prepared accurately while saving time, given the large number of microplates used. Three wells from each microplate were dispensed with only water so the background signal could be checked after the measurements. The plates were sealed with commercially available qPCR adhesive seal (Azenta: 4ti-0560). Upon sealing, the microplates were loaded into the shelves of the PlateCrane's (Hudson Robotics) plate stack.

SUPR-DSF Measurements

Within the SUPR-Suite software, the measurement plate design template was created. Settings used for the thermal denaturation measurements had the ramp rate set to 1 °C min^{-1} and was set to go from 20°C to 105°C. This meant that each plate would take ~95 mins to complete (85 mins measurement time and 10 mins to ramp back to 20°C for the next plate).

The SUPR-Suite software was used to analyse the fluorescence spectra measured. Thermal melt curves were calculated by quantifying the spectral shift (that occurs as the protein unfolds) via the barycentric mean (BCM) formula. Non-linear, least-squared regression method was used to fit a Gaussian function to the first derivative of the BCM melt curves. The peak of the Gaussian fit curves was taken as the mid-point of inflection value (T_m). The T_m values were exported for each measurement file and statistics were calculated based on the full list of 6096 T_m values.

Automated Plate Measurements

Automated control and plate handling were done via a PlateCrane and the paired SoftLinx (Hudson Robotics) software. The protocol consisted of a looped process where the PlateCrane would collect a plate from the shelf stack and place it onto the thermal block of the SUPR-DSF; the SUPR-DSF would perform the thermal denaturation measurement described above and the PlateCrane would return the plate to the plate stack. At this point, the process loops and the next plate is collected and measured. All 16 microplates were measured within a 26-hour period.

References

[1] – Gao, K.; Oerlemans, R. & Groves, M. R. Theory and applications of differential scanning fluorimetry in early-stage drug discovery. *Biophysical Reviews*, **2020**, Vol. 12(1), p. 85–104.

[2] – Krakowiak, J.; Krajewska, M. & Wawer, J. Monitoring of lysozyme thermal denaturation by volumetric measurements and nanoDSF technique in the presence of N-butylurea. *Journal of Biological Physics*, **2019**, Vol, 45, p. 161–172.

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