AAPS Interlaboratory Study

Tier 2 Method: Differential Scanning Calorimetry

Introduction

DSC is a thermoanalytical method used to measure thermal unfolding and conformational stability of a protein. It measures the difference in heat energy uptake by a sample relative to a reference during a controlled temperature change.

This protocol describes measurement of thermal unfolding of proteins by differential scanning calorimetry (DSC). The result of the measurements are reported as onset temperature (T_{onset}) and melting temperature (T_m). Only the unstressed mAbs will be analyzed on this method.

Important Notes: This protocol is not a Standard Operating Protocol and assumes the required instrument is in good working order and the analysis is performed by experienced user(s). Required calibration or check standards should be run according to the manufacturer instructions. This protocol does not contain all the details of the analysis. The analyst should rely on their best judgment, routine practices, and knowledge of the technique to conduct the study; this protocol should be used as a guideline for the analysis. Samples should be analyzed immediately after thawing and preparation.

Equipment and Materials List

- Microcal DSC instrument appropriate for small-volume sampling (< 2 mL).
- Standard recommended by the manufacturer or Lysozyme standard (1 mg/mL).
- Protein samples (at 1 mg/mL) and formulation buffers supplied by sample originators
    - Buffers and **aliquots of unstressed samples ONLY** from Shipping #1
- Appropriate dilution buffer for the mAbs
- Pipets and pipette tips as recommended by manufacturer.
- Cleaning solutions or detergents to run between samples, as recommended by the manufacturer
- Particle free water/deionized ultra-filtered water

Reagents and Solutions

Table 1: The following samples and their buffers will be provided by sample originators. Buffers and aliquots of unstressed samples from Shipping #1 should be used for this analysis. They are nominally at 1 mg/mL.

Proteins	Formulations	Samples
Amgen IgG2	10 mM sodium acetate, pH 5.0	Unstressed, 1 mg/mL
NISTmAb IgG1	12.5 mM L-histidine, 12.5 mMol L-histidine HCl, pH 6.0	Unstressed, 1 mg/mL

Procedures

Instrument Set up and Sample Preparation

1. Thaw the samples from Shipping #1 and aliquot the Tier 2 vials (for the unstressed mAb samples only) as described in the instructions into smaller aliquots for the various methods and freeze until ready to use. Be sure to follow the procedure for vigorously mixing the sample prior to aliquoting.
2. For this analysis, thaw the aliquots designated for DSC measurements by bringing them to room temperature for no more than 15 minutes. This method should only be used on the unstressed samples.
3. Turn on the instrument and supply N_2 to the system according to the manufacturer's recommendations.
4. Ensure the cleaning solvents are filled to the required volume in their designated reservoirs.
5. Set the temperature of the sample holding compartment to 5˚C to maintain the integrity of the sample prior to the experiment.
6. For concentration measurements, follow the recommended protocol described in the UV-Vis protocol. Actual concentrations are needed to normalize the data. The samples are at nominally 1 mg/mL so no dilution is required. A minimum sample volume of ~400uL will be required for each sample well.
7. Convert the concentration of protein (mg/mL) into mM by dividing by the Molecular weight in kDa - i.e. Concentration in mM = (Concentration in mg/mL) / (Molecular Weight in kDa).
8. Degas the samples under vacuum for 30 mins to get rid of microbubbles that can cause volume inaccuracy. This step can be skipped for newer calorimeter models.

Setting up a 96-well plate for Analysis

1. In a laminar flow biocontainment cabinet, load the samples (unstressed only) and their respective buffers in pairs into 96 well plates. The analysis of each sample is performed by comparing the thermal profile of that sample against its reference (buffer). The source well for each of these solutions has to be specified for each sample to be analyzed. This is the general sequence of runs: 1) water, 2) standard, 3) samples, and 4) a final clean. There is an intermediate cleaning step between each of these injections.
2. The following runs are recommended:
   a. Lysozyme at 1 mg/mL (in water) can be used as a standard for system suitability and run before and after every run. You may also use vendor recommended standard(s) for system suitability.
   b. A contrad-contrad cleaning sample set is run after every three samples, followed by two water sample sets to ensure all the detergent is out of the system
   c. Buffer-buffer scans verify the suitability of the instrument prior to sample measurement (i.e. assessment of instrumentation error) as well as establish a baseline; while water scans are run to clean the cells.
3. Cover the well plate before taking the plate out of the Biosafety Cabinet to avoid sample contamination.
4. Place the plate in the sample holding compartment in the proper orientation.

Sample Measurements

1. Review the **DSC Data** template for the requested information before beginning the measurements and input the set-up information.
2. Depending on the instrumentation, samples can be loaded into the cell manually or automatically. The autosampler is preferred.
3. In the acquisition software, enter the sample information in the order the plate was loaded. Enter sample concentration values into the software.
4. Ensure that an automated cleaning cycle is set to run between samples so that cells are cleaned with detergents before every sample scan, which should be followed by multiple water rinses steps to ensure no detergent residue is left in the cells.
5. Ensure that the instrument parameters are set to the following as shown in Table 2 below. It is important to keep the scan rate at 120°C/h. Scan rates should be kept the same to obtain comparable data between samples. Variation in scan rate can lead to variation in the T_m and melting profile of the molecule of interest.
6. Specify 25°C for Auto-Fill Cell temperature. This value should be higher than the starting temperature and ensures that sample analysis begins after the temperature ramp has been initiated.
7. Prior to initiating analysis, ensure all solvent reservoirs are full and periodically monitor solvent volume during the run to ensure that there is enough volume for all of the rinse/wash cycles throughout the run.
8. Initiate the run sequence.

Table 2: Recommended Scan Parameters

Starting Temperature (°C)	20
Final Temperature (°C)	110
Scan rate (°C/h)	120
PreScan Thermostat (min.)	5
Post Scan Thermostat (min.)	2
# of rescans	0 (for samples); 2 or more (for buffers being scanned for the first time)
Filtering period (sec.)	10
Feedback Mode/Gain	Low
Post cycle Thermostat (°C)	20

Data reporting

1. Data analysis should be performed using the software provided with the instrument.
2. Subtract baseline and buffer scans from the sample scans and normalize to the concentration in the units kcal/mole/ºC.
3. Using the software, pick the apparent Tms of the sample. Note the Tm onset, apparent ΔH (enthalpy) and apparent Tms.
4. If samples show peaks with more than one transition Non 2 state curve fitting can be used to model the peaks.
5. Report the curve fit Tm's and curve fit enthalpy in the **DSC Data** reporting template**.**

   Note: Post peak aggregation can be misinterpreted as domain unfolding. In these cases samples can be run at low concentration (0.2 mg/ml) and compare it with the thermogram of high concentration (1 mg/ml). Decrease in the signal intensity of the aggregation peak confirms its identity.

References

1. Durowoju, I. B., Bhandal, K. S., Hu, J., Carpick, B., Kirkitadze, M. Differential Scanning Calorimetry-A Method for Assessing the Thermal Stability and Conformation of Protein Antigen. JOVE, 2017, 1-8.
2. Malvern Instruments. Characterization of Bioparmaceutical Stability with Differential Scanning Calorimetry: Candidate Selection for Developability. 2017.

Further Information

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