MDH Characterization Protocol:

SYPRO ORANGE Fast Thermal Melt

Fluorescence-based thermal shift assay (FTS) assay to assess ligand binding - *Protocol adapted from Dr. J. Bell, 2021.*

As proteins unfold, the hydrophobic residues typically buried within the interior become exposed to the aqueous environment. To monitor the exposure of these hydrophobic residues, the binding of a hydrophobic fluorescent dye (SYPRO Orange) is quantitated as a function of the protein unfolding. The SYPRO Orange dye is highly fluorescent upon binding to hydrophobic sites on unfolded proteins. Typically in a Fluor-based thermal shift assay, the thermal denaturation of a protein is marked by a change (increase) in fluorescence intensity as the protein unfolds, generating a sigmoidal curve that can be described by a two-state transition.

Once this thermal shift is defined for a protein, this characterization can be used to monitor alterations to a protein's structure, such as ligand interactions, that would modulate the thermal melt behavior of the protein.

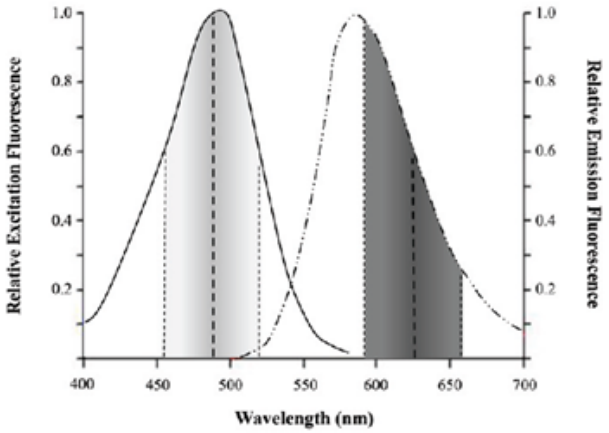

Figure 11. Excitation (solid) and emission (dashed) spectra of SYPRO orange fluorescent dye in BSA. Shaded areas represent the relative range of light transmitted through the BioRad 485 ± 30 nm excitation and 625 ± 30 nm emission filters used in this experiment. This figure is modified from Invitrogen Life Technologies product information (Cat. No. S-6650) and copied from Biotechniques (2012) 53:231-238.

HINTS:

Protein of interest (POI) should be dissolved in a minimal buffer that supports its stability (like CD, you can use 5 mM phosphate buffer – if you are not testing metals as an additive!!! Alternatives – HEPES. If you are testing pH or buffers, try to keep your protein in a low concentration buffer 5-10 mM; if pH or buffer salts are not being tested, 50 mM should be fine.)

200X stock of SYPRO Orange (Note: The manufacturer provides SYPRO Orange as a 5000X stock in DMSO. You should make a dilution to 200X with DMSO in an amber tube to protein the SYPRO orange from light. Your final concentration in the tube or well will be 20X.) Thus you will use 2.5 µl of 200X per 25 µl assay (or 8 µl in a 80 µl assay to get to the final 20X concentration).

96 well plate with sealing tape – plate should not be clear (opaic white work well), conical is best. Often the same plates as used for real time PCR

A general **final** protein concentration in the reaction should be ~ 4 µM but this may change based on protein and experimental question.

The final volume in each well will be 25 µl.

1. You should set up your rxns in microcentrifuge tubes. *You should complete each reaction in triplicate.* Therefore, in your microfuge tubes you should set up each condition in a total volume of 80 µL, that you will aliquot at 25 µL per 3 wells.
    a. 8 µl of 200X dye
    b. Up to 72 µl protein or ligand total

2. For each condition, you will need a control (no protein of interest) condition to examine the SYPRO Orange reaction with the condition. ***Fill out an experimental setup worksheet to aid in mapping out your reaction setup.*** Setup your reactions such that you do not pipette any volumes less than 1 µl.

3. Order of reagent addition to tubes: 1) Starting buffer, 2) Condition being tested, 3) SYPRO Orange, 4) Protein of Interest. Add your POI last so that the condition being tested and SYPRO Orange are near their final concentrations. Otherwise, your POI will be exposed to a higher concentration of the condition or % of DMSO (SYPRO Orange solvent) than what you accounted for in your experimental plan… a big OOPS! ***SYPRO Orange final conc = 20X; protein final conc = 4 µM***

4. Transfer to a 96 well plate. Make sure that you have a plate that is compatible with the instrument and the measurements that you want to complete.
   a. After you have aliquoted your 25 mL into each well, seal the plate.
   b. Remember you are taking measurements, so minimize fingerprints, smudges on plate that will obscure measurements.
   c. Spin the plate in a swinging bucket centrifuge on the appropriate plate holders inserts (with an empty plate acting as a balance!) at 1000 rpm for 1 minute. This will remove air bubbles at the bottom of the well and ensure the reaction is at the bottom of the well. DON'T spin longer! You can separate the DMSO (containing your SYPRO Orange) from the aqueous phase (containing your POI)

5. The thermal scans will be performed from 10 to 95°C at 0.5°C increase/minute and fluorescence scans collected every 30 s.

6. On the BioRad system, make sure that you have chosen the FRET channel for data collection.
   a. NO – you can't get the data after the fact no matter how many "other" channels you collected.

7. After the run, examine the triplicates. If your triplicates are similar, average data. If not, evaluate if you have good quality data.

8. Subtract averaged "No Protein" + condition control as appropriate from your averaged POI + condition.

9. Plot the corrected fluorescence intensity versus temperature.
   a. Examine the shape of the melting curve. What does it tell you about your protein of interest under conditions tested?
   b. From these plots, calculate the melting temperature, Tm. To calculate your Tm value, you can fit the sigmoidal portion to a 4-parameter sigmoidal, or take the 1st derivative of your data where the maximum will be the inflection point of the sigmoidal curve or take the 2nd derivative of your data where the minimum will be the inflection point of the sigmoidal curve.
   c. Compare the Tm for your protein without additives to protein plus different conditions. What conclusions can you draw with respect to the protein:condition and protein stability? Does stability correlate to the condition?

No Protein Control (NPC) everything but the protein… IF the signal is high, there is likely contamination

Liquid Only Control (LOC) – any ligand (not protein) dye and buffer/water. If there is a dye ligand interaction it will show up here as a melt or high background signal

Protein:Dye ratio – some manufactures suggest using 5 or 8X dye. Hold protein concentration steady and change the final conc of dye. The reciprocal experiment should also be run at two dye concentrations.

High initial Background: Native protein has external hydrophobic residues binding to protein. This may mean the protein of interest is not suitable for FTM. One option might be to optimize the protein to dye ratio.

Flat signal or decrease in signal: Protein may already be partially or fully denatured. This type of curve is due to protein aggregation or if the protein is partially unfolded. Ensure fresh native sampled is used. Perform a buffer screen to find a condition that maintains the protein in a stable state.

Curves with low relative florescence levels: Low protein concentration. Perform protein:dye titration studies.