Thermal Shift Assay Protocol – Partch Lab
(Using an Applied Biosystems ViiA 7 qPCR machine)

This protocol is designed to screen protein stability in a series of different buffer conditions. Alternatively, you can screen different small molecule additives or drugs, or screen different proteins in a set buffer.

<u>In 96-well format, each well has:</u>
10 µl 5-20 µM protein (diluted in water or ~5 mM buffer/50 mM salt*)
10 µl 5X Sypro Orange dye (diluted in water)
20 µl water
10 µl 5X buffer stocks from deep-well block

1. Fill a 96-well deep-well block with ~2 ml of 5X concentrated buffers of your choice. You can survey buffer identity/pH, salt concentration/identity, small molecule additives, etc. To optimize for conditions in cryo-probed NMR experiments, we typically use several buffers in a pH range from 5.0 – 7.5 with several concentrations each of low salt. We store our deep-well block at 4°C sealed with adhesive foil and bring it up to room temperature before use. Following a quick spin on a benchtop centrifuge, carefully remove adhesive to prevent mixing of buffers.
2. Dilute protein to be tested to 5 µM in water or dilute buffer* in order to sample the assay buffer conditions more effectively. For one 96-well plate, we make 1.3 ml.
3. Dilute Sypro Orange dye [Sigma S5692] to 5X working stock in water (final: 1X). For one 96-well plate, we make 1.3 ml.
4. Make 5 ml master mix of water, protein and dye (enough for 125 wells). Put into a reagent reservoir and use a multichannel pipettor to add 40 µl master mix into each well of the 96-well plate. You'll need to use a plate appropriate for the qPCR machine you are going to use. For the AB ViiA 7 machine, we use Cat # 4346907, MicroAmp Fast 96 reaction plate, 0.1 ml.
5. Using the multichannel pipettor, add 10 µl buffer stocks from the deep-well block into the appropriate wells.
6. Seal carefully with optically clear adhesive. We use Bio-Rad Cat #MSB1001, but you can use any optically clear sealing tape.
7. Turn on the ViiA 7 qPCR machine and insert Fast 96 plate into plate holder.
8. Open up the ViiA 7 RUO software and select Experimental Setup: follow instructions for the setting on the left tab below:
   a) Experiment Properties
      i) Set up – Fast 96-well block (0.1 ml)
      ii) Experiment type – MELT CURVE
      iii) Reagents used to detect target sequence – OTHER
      iv) Ramp speed – STANDARD

b) Define
    i) Target name – TARGET 1
    ii) Reporter – ROX
    iii) Quencher – NONE
    iv) Passive reference – NONE
c) Assign
    i) Highlight all 96 wells
    ii) Check mark "Target 1" to the left of the plate layout
d) Run method
    i) Delete Step 2
    ii) Change to "Step and Hold 1:00"
    iii) Set temperatures -- 25°C (2:00 initial equilibration) ramping up in increments of 1°C to a final temperature of 95°C (2:00 hold).
    iv) Highlight all three cameras
    v) Select total volume in well – 50 µl

9. Click on the "RUN" tab to the left. Wait to make sure that your run won't have a time out error – if it moves past the 2:00 incubation at 25C, you're set.
10. Once the experiment is done, export data and save the Excel file. You may want to save the melt curve image plot generated by the ViiA 7 software by clicking on "Save to image file".
11. Open raw data in Excel and copy data to new sheet. Organize data in columns with fluorescence from each well alongside temperature. Copy organized data to a new sheet. Generate an Excel macro with the text below [Note: you can re-use this Excel file once you've generated the macro and just copy/paste data in]. Truncate post-peak fluorescence points using the 'deleteaftermax' macro.
12. Export the truncated datasets to Graph Pad Prism and do non-linear fitting to a melting curve to fit for the T_m (see below).