**ITC for the study of CDF proteins CTDs’ interaction with divalent transition metal cations (using low-volume Nano ITC calorimeter by TA Instruments)**

1. Degas all your samples – put the Eppendorfs (protein, metal, buffer solutions) with their lid open in the appropriate place in the degassing station, then close the plastic lid of the station and press the “Timer” button. It should be defined as 15 minutes, if not change for 15 minutes and start. Notice that the temperature is 25 °C (or whatever temperature you are going to use in your measurement), if not, change the temperature using the “Temp” button.
2. Sign in with your user to the ITC computer (BGU-USERS\username).
3. Register to your account in the ITC computer.
4. Open the nano ITC LV (power button in the back of the instrument), follow the instruction window that pops up in the computer. Notice that the calibration of the injection lid (where you place the titrant/ligand syringe) was finished so it can accommodate a full 50 µL syringe.
5. Open the ITCRun software, and setup the experimental conditions. For MamM CTD, these were the defined conditions:
   1. Incremental titration injection with 20 aliquots, the first of 1.25 µL and the next 19 aliquots of 2.5 µL each, with injection intervals of 350 sec (write it once and drag down to copy for all aliquots)
   2. Temperature = 25 °C (press “apply” or it won’t be changed!)
   3. Stirring rate = 250 RPM
   4. Syringe size = 50 µL
   5. Save experiment every 10 minutes
   6. Initial and final baselines = 180 sec
   7. Syringe concentration = 1.4 mM
   8. Cell concentration = 0.050 mM
6. Wash the instrument using the degassing station. Insert the cleaning needle into the cell and the tube into the solution in which you want to wash the system with (first detergent, then DDW, then MQ and finally with your buffer). Press the “Vac” button and then “Clean”. Use at least 20 mL of each wash solution. Move the pump directly from each wash solution to the next in line (wipe the tube after using the detergent). When finished, press the “Clean” button again to stop the pump.
7. Wash the 500 µL (cell-filling) and 50 µL (titrant) syringes. Insert the needle into the tube until it is blocks the tube so it allows vacuum, take off the plunger and put the syringe in the washing solutions the same way you put the tube in the previous section, and wash using the same procedure (for the cell-filling syringe use at least 10 mL of each washing solution, and for the titrant syringe use at least 5 mL).
8. Clean the plungers using the same solutions (just insert it to each solution and wipe if afterwards, 3 times in each solution).
9. After all parts are washed, use the cell-filling syringe to take off buffer leftovers from the cell.
10. Pull in and out the plungers from the syringes so to verify that they contain no buffer (you cannot see it in the needle so just do it until no more buffer splashes).
11. Fill in the cell-filling syringe with 320 µL of your 50 µM protein solution (after degassing is finished), avoid bubbles. Fill the cell with 300 µL solution (press the plunger slowly, not until the end but until it reaches ~ 20 µL if you filled it with 320 µL).
12. Fill the titrant syringe with 50 uL of your 1.4 mM metal solution in the same buffer, avoid bubbles. To avoid the air trapped right near the plunger, take the plunger out, let the solution flow down until it reaches the end of the syringe and the bubble is out, than insert the plunger and push it until the solution starts to get out of the needle. Then, refill the syringe with a bit more than 50 µL metal solution (verify no bubbles). Wipe the syringe and screw it into its cell, notice that when you tight the screwing, a drop should comes out of the syringe (because of the overfilling). Wipe the syringe again and place it in the ITC cell.
13. Start the stirring.
14. Start data collection. Save the file in your folder under Users/Zarivach/Name and name the file as you wish.

\*One titration takes about 2.5-3 hours;

\*\*First titrat metal into the protein solution, then metal into buffer (control), and if you don’t need to change the experimental parameters, then make at least 2 more metal to protein titrations (at least 3 overall).

\*\*\*In between titrations using the same titrant, wash the cell and the cell-filling syringe with DDW, MQ and buffer (no need to wash the cell-filling syringe after it was used for control titration).

1. In the end of the experiment, wash the cell, cell-filling syringe and titrant syringe with detergent, DDW and MQ. Closed the system (the ITC instrument, the degassing station, the ITCRun software and register off your payment user and your computer user).
2. Analyze the data using TA NanoAnalyze Data Analysis software. The data for MamM CTD of each measurement were fitted to independent model combined with blank constant model, and the thermodynamics values are the average of the three different titrations.