Biophysical
Instrumentation
Facility

MICROCAL Isothermal Titration Calorimeter

1.	If you have no idea of your system's binding constant, suggestions for starting concentrations of protein and ligand solutions are given in Section 6.5 of the VP-ITC User's Manual. (available online at www.microcal.com)

2.	Protein and ligand solutions need to be in the same buffer so no heat of dilution will be generated when they mix. If possible, dialyze protein and ligand solutions against the buffer they're in and use the dialysis buffer in the reference cell. The Sample Preparation Tips section of the ITC's webpage discusses several important parameters. DTT and TCEP are better avoided in your buffer. See Section 6.3 of the User's Manual for reasons and possible substitutes.

3.	Turn on the computer first, then turn on the instrument itself. Switch is a rocking toggle located at the upper left on the back. Open the MicroCal VP-ITC software about a minute after turning on the instrument. Otherwise, there won't be a Y-axis scale chosen under the Setup/Maintenance tab or constants loaded under the Constants tab. Just try again if it fails the first time.

4.	Before you start degassing your samples, get the ITC moving toward your desired working temperature. Go to the Thermostat/Calibration tab and adjust the temperature there. Click Set Jacket Temp to set it. The ITC's range is 2° to 80°.

5.	Solutions need to be degassed first so that bubbles don't form and interfere with heat transfer. Turn on power to the ThermoVac with a rocking toggle switch at the upper right on the back. Set the temperature of the ThermoVac unit to 3-4° below your experimental run temp. since it's much quicker for the machine to heat the sample a little than to cool it. (Note: there is a missing decimal point on the temperature display. 24° looks like 240°.)

Put 2.5 ml each of protein solution and your buffer into plastic vials with stir bars and put ~800ul of ligand solution into a small glass tube. All can be degassed at the same time. Place the vacuum cap over the vacuum chamber and tighten the silver bleeder valve on top of it to increase vacuum. Watch for foaming of the solutions at first and reduce vacuum (by turning the bleeder valve counterclockwise) to calm that as necessary. Degas for 10 minutes.

6.	Open ITC Controls window. Set Experimental Parameters as follows:

Total # injections   up to 500
Cell temperature   actual temp. you want
Reference Power   15 (middle of the range)

        Initial delay   at least 60 sec
        Syringe conc.   conc. of ligand (can add this later)
        Cell conc.   conc. of protein (can add this later)
Stirring speed   307 is recommended (suspended
                                 particles will require higher speed)
        Data file name   your choice.itc (unique characters in
    first ten)

        Feedback Mode   high (for faster response times)
ITC Equil. Options   auto (machine will proceed on its
own through equil. steps)

    Set Injection Parameters as follows:

Volume   10 ul, maybe (see special injection
                                 notes at the bottom of this SOP)
    Duration   20 sec (default is always 2X volume to allow time for return to baseline)
    Spacing (between injections)   240 (typical range 180 - 300)
    Filter period   2 (higher for slow thermal process)

    Edit Mode
    Can make different settings for first injection with Unique button

7.   If it has been a week since degassed water was put in the reference cell, load the reference cell with 1.8 ml of degassed water. Place the needle all the way at the bottom of the chamber then withdraw it back up ~1mm, leaving it in place as you slowly add the water. (i.e., don't withdraw the needle as you fill) Once the water comes up out of the tube, flush any bubbles out by using small bursts of water pressure from the syringe. Then stir the water in the chamber as much as you can with the needle to dislodge any air bubbles from the shoulders of the chamber. Once bubbles are gone, remove excess volume from the cell by placing the delivery needle on the cell's inner ledge and draw fluid out until you pull air out.
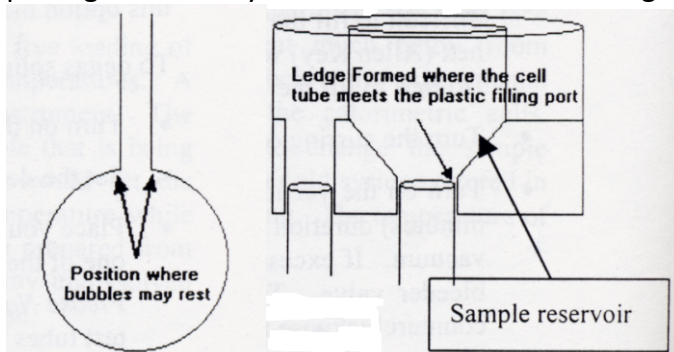
8.	After rinsing the sample cell twice with ~2 ml of your buffer, load 1.8 ml of your protein sample into the sample cell as in Step 7.

9.	To load your ligand into the ITC syringe, first attach the tubing of the loading syringe to the silver filling port. Click Open Fill Port on the lower right of the ITC Controls window. Place syringe paddle in the tube of your degassed ligand with the tip almost at the bottom. Pull on the loading syringe to draw your ligand up into the ITC syringe. When the syringe is completely full and some ligand has come out into the loading tubing, click Close Fill Port. If there is a bubble at the very top of the ITC syringe, ignore it even if it seems large. MicroCal has shown that a bubble just below the plunger tip does not affect injection accuracy.

10.	To check that there are no bubbles in the ITC syringe along with your ligand, remove the loading syringe tubing and run the Purge-Refill function once or twice. If there are any bubbles, you'll see them pop up in the ligand tube.

11.	Remove the syringe from its holder and carefully wipe extra liquid off the paddle with a Kimwipe. DO NOT EXPEL LIQUID from the bottom of the paddle (which we used to do with the older syringe). This will erase the syringe's calibrations and will require a service call to reset them. Program a tiny first injection to deal with a possible bubble at the bottom of the paddle. (See Injection Notes at the bottom of this SOP.)

12.	 Carefully insert the pipette into the sample cell access tube, the hole on the right. Near the bottom you have to push a little against a rubber O-ring to seat the assembly. Be sure the syringe is seated properly, pushed all the way down.

13.	Before you start your run, set your data path on the Setup / Maintenance tab by double-clicking on the word data in the Data File Path box. This will open the Data folder and you can make a folder for yourself. Type the name of the folder (or subfolder) in the Folder Name box, then click the Add Folder button. Once the folder is created, find it in the list and double-click on it to open it. Click OK to set the data path.

14.	Click on the Start flag to start the run. When the Jacket and Sample temperatures have reached your set point, the red status letters change from "Heating" to "ITC Pre-run". The machine will then go into "Pre-stirring equilibration" mode. If it stays there for 20-30 minutes, hit Stop then Start again quickly and it will cycle through all its equilibrations but faster this time.

15.	When it reaches "Final baseline equilibration" the stirrer will start. If at this point the lower right window (pipette control) goes gray, it means the syringe is not communicating so check the wire connection between the syringe and the box. You may need to wiggle that to make the connection and start again.

16.	The next stage will be "ITC Pre-titration Delay". You can click on the Rescale to Show All button to see your whole trace. Finally, you will get to "ITC injection #1" and the experiment is off and running. You can calculate the approximate running time by multiplying your number of injections times the spacing time interval.

17.	If you think you have enough injections, you can stop the ITC <u>in between</u> <u>injections</u> by clicking on the STOP sign on the ITC Controls tab. You can also let it finish all the injections you entered in the Method, i.e., let it keep injecting until it runs out of ligand.

18.	To analyze your data, click on the MicroCal Analysis Launcher icon. Double click on VP-ITC and this will open a second version of Origin not interlaced with instrument control. Follow the instructions in the **Analyzing ITC Data** protocol on the website.

19.	To clean up the machine for the next user, remove your sample and rinse the sample chamber with water. Then hook up the automatic washing system and put the injector in the loop so that detergent solution will pass through it as well as the sample chamber. Pull 250 ml of 5% Liqui-Nox through everything followed by an equal volume of water. Leave water in the sample chamber. Rinse out the injector with 2 ml 100% methanol (by hand) then pull air through it for 10-15 minutes using the house vacuum set-up. Leave the paddle in air.

20.	To close the VPViewer software, pull down from System and choose Quit Program. Closing the window from the X in the upper right-hand corner will result in your getting stuck in an infinite loop. Once the software is closed, you can turn off the ITC with the switch on the back. Leave the computer on.

Injection Notes: You can set your first injection to be 0.5 ul. This will expel the tiny bubble that may form at the tip of the syringe when you insert the syringe into the sample cell. This way your first full-size injection (the second one) will be more accurate. Delete the data from the first tiny injection before doing curve-fitting in Origin.

You should note how long it takes for the baseline to be reached again after the first tiny injection and program future runs to include that delay.

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> Note: This protocol is a practical hands-on guide to using the instrument.
> Reading this is not a substitute for reading the manufacturer's manual,
> something we strongly recommend that you do.