IOWA STATE UNIVERSITY
Chemical Instrumentation Facility

1234 Hach Hall
515-294-5805
www.cif.iastate.edu

Microcal VP-ITC Operating Instructions

10/18/2012 S.V. draft

Location: **1238 Hach Hall**
Contact: **Steve Veysey, 1234 Hach Hall**

SAFETY

All researchers working in 1238 Hach Hall must complete the EH&S course: *"Fire Safety and Extinguisher Training"*. When preparing samples in this room, please wear all appropriate personal protective equipment. Aprons, safety glasses, and rubber gloves are available in 1238A Hach Hall. All use of solvents should occur in 1238A Hach Hall.

Properly dispose of glass pipettes and plastic pipette tips in the containers provided. Waste solvents can be disposed of in the waste containers provided in 1238A. All of the computers in this lab have direct links from the desktop to MSDS sheets, the EH&S Laboratory Safety Manual and to the CIF Safety Manual.

INTRODUCTION

The MicroCal VP-ITC (Isothermal Titration Calorimeter) unit directly measures heat evolved or absorbed in liquid samples as a result of mixing precise amounts of reactants. A spinning syringe is utilized for injecting and mixing of reactants. The user inputs the experimental parameters (temperature, number of injections, injection volumes) and the computer carries out the experiment. Origin® software is then used to analyze the ITC data using fitting models to calculate reaction stoichiometry (n), binding constant (KD), enthalpy (ΔH) and entropy (ΔS).

This document presents a training guide prepared in 2006 by former ISU student Dr. Gulden Camci-Unal. Minor procedural changes are annotated and summarized at the end.

by Gulden Camci-Unal

Methods used to study molecular interactions

- *Traditional enzyme assays:*
  - Use probes to monitor substrate degradation or product formation
    - Probes are system dependent
    - Need to be optimized for each specific case
  - Substrate modification might be required
- *Optical methods:*
  - Detection system is very sensitive to the experimental conditions[1]

[1] http://www.microcalorimetry.com/index.php?id=316

by Gulden Camci-Unal

Isothermal Titration Microcalorimetry (ITC)

- Biomolecular interactions
  - Heat is absorbed
  - Given off
- Monitors enzyme kinetics
- Measures thermodynamic parameters[1]

[1] http://www.microcalorimetry.com/index.php?id=316

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ITC (Cont'd)

- ITC can monitor
  - Protein-DNA
  - Protein-protein
  - Antibody-antigen
  - Enzyme-inhibitor
- Experiments can be done @ different
  - Temperatures
  - pH
  - Buffer conditions
- ITC data is used for characterization of
  - Structure. activity, fuction of
    - Proteins, nucleic acids, lipids, etc.[2]

[2] http://www.microcalorimetry.com/index.php?id=317

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Uses

- ITC measures
  - Binding constant
    - K_b
  - Stoichiometry of binding
    - n
  - Thermodynamic parameters of binding
    - ΔH
    - ΔS
- $\Delta G = \Delta H - (T \times \Delta S)$

by Gulden Camci-Unal

Advantages

- No labeling (e.g. chemical tags)
- No immobilization required

- Turbid solutions
- Colored solutions
- Particulate suspensions can be experimented[3]

- No molecular weight limitation
- No molecular weight dependent sensitivity[4]

[3] http://www.microcalorimetry.com/index.php?id=13
[4] http://www.microcalorimetry.com/index.php?id=64

by Gulden Camci-Unal

Advantages (Cont'd)

- Fast analytical technique
- Heat of binding is directly measured[5]

- Sensitive
- Non-destructive[1]

- Small amount of sample required

[1] http://www.microcalorimetry.com/index.php?id=316
[5] http://www.microcalorimetry.com/index.php?id=13

by Gulden Camci-Unal

A typical ITC experiment

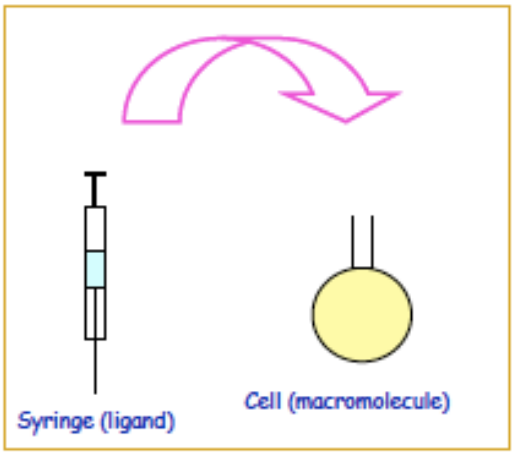
Titration of ligand into macromolecule

Syringe (0.301 mL):

- ligand
- buffer

Cell (1.4288 mL):

- macromolecule
- buffer



∑ # of injections:
Syringe conc: mM
Stir speed: rpm
Spacing: sec

Cell temp: °C
Cell conc: mM
Injection volume: µL

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Generic plot for an ITC experiment[5]

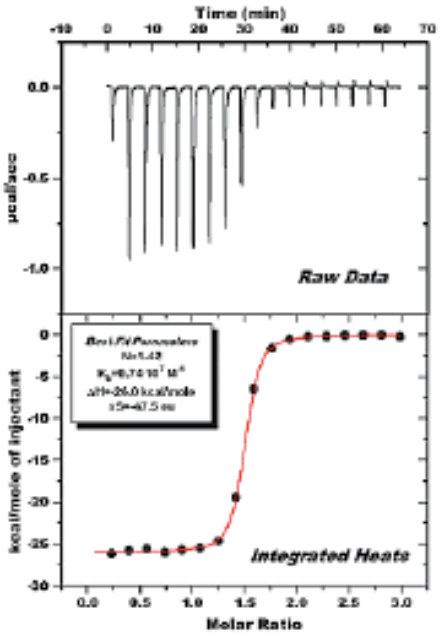

Figure adopted from [5] http://www.microcalorimetry.com/index.php?id=13

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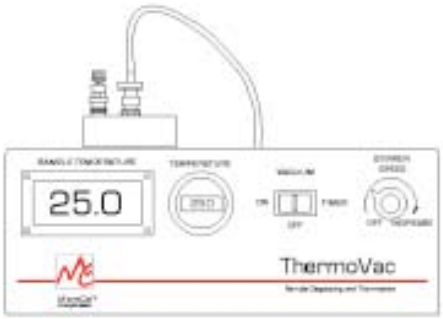
ITC Instrument[6]

- Composed of 2 parts:
  - 1. VP-ITC computer controller
  - 2. ThermoVac degassing station[6]



Pictures adopted from [6] VP-ITC MicroCalorimeter User's Manual, p. 3 and 11

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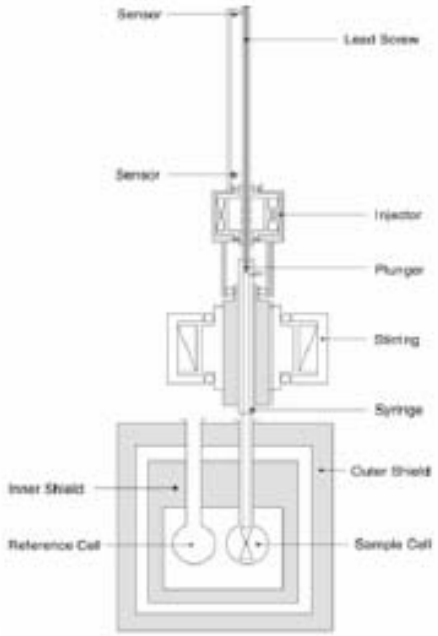
ITC Sampling Unit[6]



Pictures adopted from [6] VP-ITC MicroCalorimeter User's Manual, p. 2 and 3

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Principles[6]

- Exothermic reaction: produces (-) change in differential power
- Endothermic reaction: produces (+) change in differential power

- Entire experiment is computer controlled
- Released or absorbed heat is directly proportional to binding strength
- Once macromolecule is saturated w/ ligand, heat signal decreases

- *Data analysis:* Origin Software

[6] VP-ITC MicroCalorimeter User's Manual, p. 2

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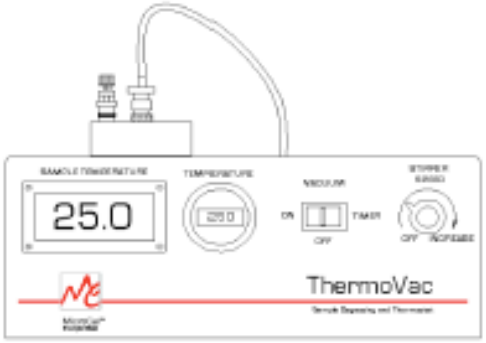
ThermoVac Unit[6]

- To degas samples
- To clean the cells
- Can thermostat 0-80°C
- Pulls a vacuum about 28.4 in of Hg
- Stirs the sample w/ magnetic stir bars while degassing[6]



Picture adopted from [6] VP-ITC MicroCalorimeter User's Manual, p. 11

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VPViewer

- Temperature: °C
- Differential power: µcal/sec
- Differential temperature: °C
- Reference power: µcal/sec
- Syringe concentration: mM
- Cell concentration: mM
- Stir speed: rpm
- Injection volume: µL
- Duration: sec
- Spacing: sec
- Filler period: sec

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Operation ranges of experimental parameters[6]

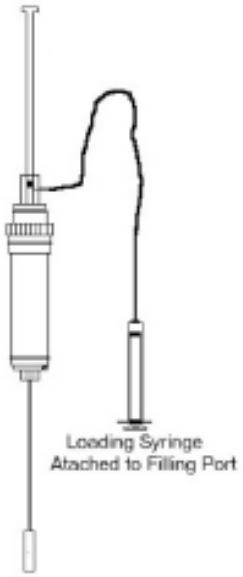
- Binding constants
  - 10^2 – 10^9 M^{-1} (Ref. 7)
- Temperature
  - 2°C – 80°C (Ref. 6)
- Concentration of ligand ≈ (7 to 10) x (Concentration of macromolecule)[4]

[4] http://www.microcalorimetry.com/index.php?id=64
[6] VP-ITC MicroCalorimeter User's Manual, p. 11
[7] http://www.microcalorimetry.com/index.php?id=312

by Gulden Camci-Unal

Precautions

- 3 types syringes:
- *Instrument syringe:*
  - Handle it very carefully
    - Must NOT bend!
    - Otherwise baseline stability is lost
    - Permanent deformation
    - Improper operation
    - It might be unusable for future experiments
- *Filling syringe:*
  - Do NOT hit the needle against any object
  - Remove air bubbles from the filling syringe
- *Loading syringe*
- VP-ITC cell material:
  Hastelloy® C-276
  - Strong acids should NOT be used[6]



[6] VP-ITC MicroCalorimeter User's Manual, p. 2
Picture adopted from [6] VP-ITC MicroCalorimeter User's Manual, p. 49

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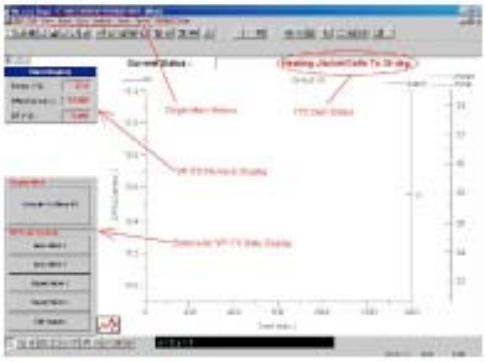
Procedure for ITC Experiments

- 1- Turn on the instrument
- 2- Click VPViewer
  - Two programs start:
    - One gives the parameters for the system
    - Other plots data against time



Pictures adopted from [6] VP-ITC MicroCalorimeter User's Manual, p. 17 and 31

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Procedure for ITC Experiments

- 3- Make sure the run light is ON
  - If not;
    - Close the VPViewer program
    - Turn the power switch of VP-ITC off
    - Wait for couple of seconds
    - Turn the power switch of VP-ITC back on
    - Open the VPViewer program again

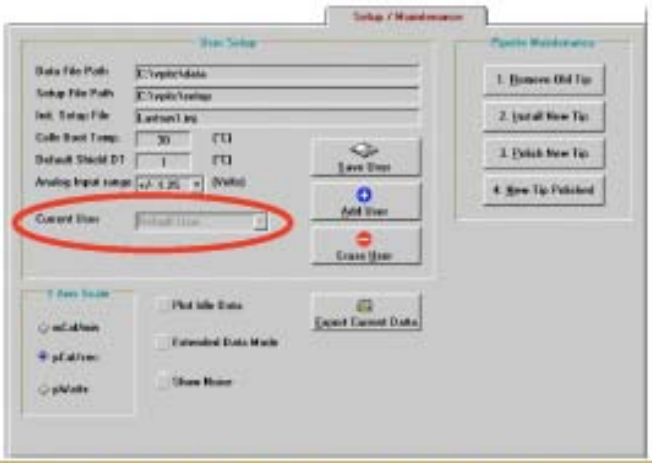
Picture adopted from online version of " VP-ITC MicroCalorimeter User's Manual, cover picture

by Gulden Camci-Unal

Procedure for ITC Experiments (Cont'd)

- 4- Change the current user name
  - Go to Setup/Maintenance
  - Pick "Gulden" from ITC users list

Note: Pick YOUR username from the ITC users list



Picture adopted from " VP-ITC MicroCalorimeter User's Manual, p. 21

by Gulden Camci-Unal

Procedure for ITC Experiments (Cont'd)

- 5- Set the temperature for your experiment

- 6- Prepare 1 mL of ligand and 2 mL macromolecule (prepare 2 macromolecule solutions which are exactly the same, use one of them to rinse the sample cell before actual sample loading)

Picture adopted from ° VP-ITC MicroCalorimeter User's Manual, p. 26

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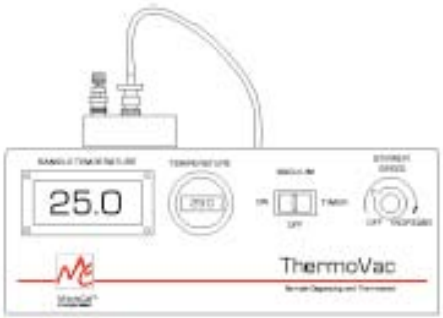
Procedure for ITC Experiments (Cont'd)

- 7- Degas your ligand and macromolecule both @ 20°C
  - ALWAYS turn the stirrer on and then vacuum on (vacuum is turned on by pressing the TIMER switch)
  - Turn the stirrer off before releasing the vacuum
  - Release the vacuum
  - Then increase the temperature 1°C below your experiment temperature (in order NOT to get your sample evaporated)

** If foaming occurs during degassing: REDUCE vacuum!



Picture adopted from ° VP-ITC MicroCalorimeter User's Manual, p. 11

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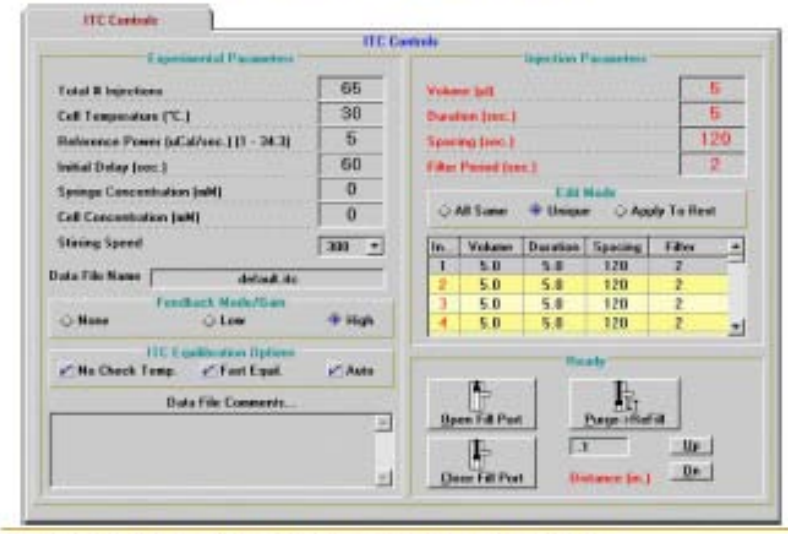
Procedure for ITC Experiments (Cont'd)

- 8- Enter experimental run parameters



Picture adopted from ⁶ VP-ITC MicroCalorimeter User's Manual, p. 21

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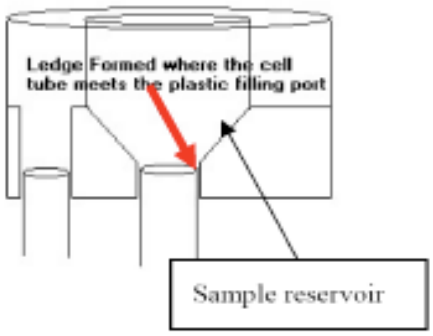
Procedure for ITC Experiments (Cont'd)

- 9- Rinse the sample cell w/ the same macromolecule solution that you will use
- 10- Load the sample cell w/ your macromolecule
  - Place the sample syringe on the ledge
  - Withdraw any excess solution
- 11- Close the sample port



Picture adopted from ⁶ VP-ITC MicroCalorimeter User's Manual, p. 42

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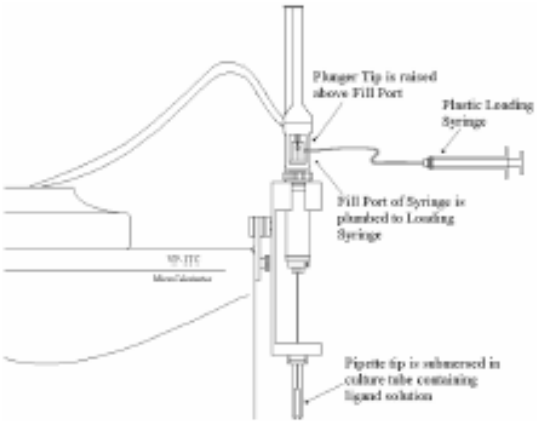
Procedure for ITC Experiments (Cont'd)

- 12- Load the syringe w/ your ligand solution
- 13- Withdraw the ligand solution VERY slowly until you see the solution about 1 cm after the exit of the filling port and click "Close fill port". Then remove the plastic tubing form the syringe



Picture adopted from ⁶ VP-ITC MicroCalorimeter User's Manual, p. 43

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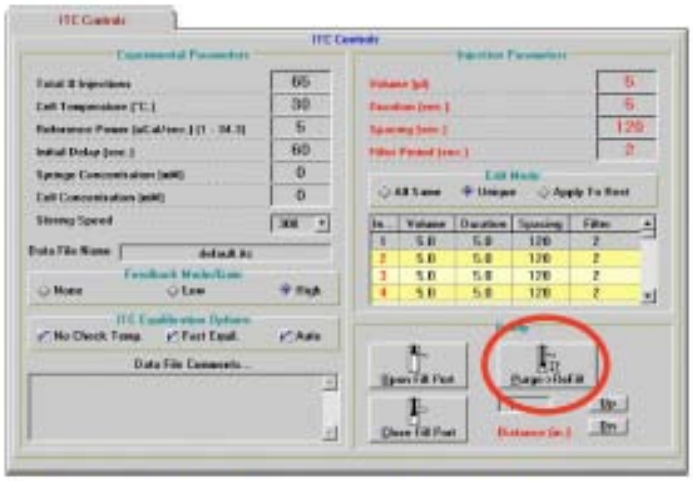
Procedure for ITC Experiments (Cont'd)

- 14- Purge and refill 3 times



- 15- Open the sample port and insert the syringe into the sample cell

Picture adopted from ⁶ VP-ITC MicroCalorimeter User's Manual, p. 21

by Gulden Camci-Unal

Procedure for ITC Experiments (Cont'd)

- 16- Tell the program to go!

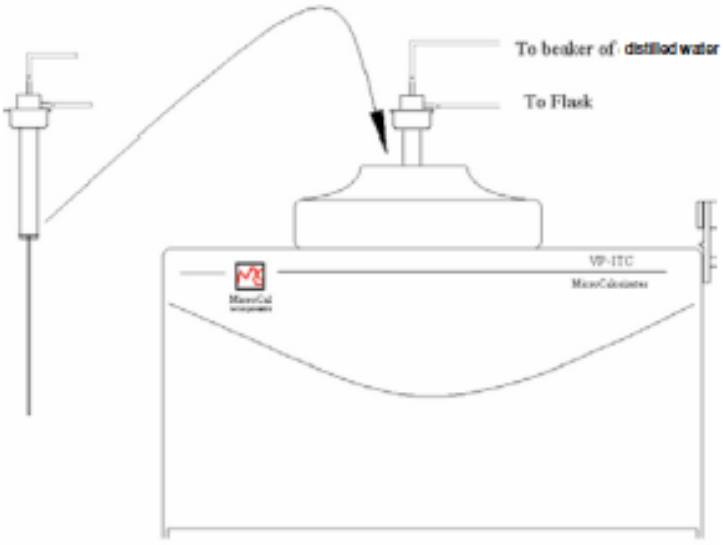
** When the experiment is done, put the instrument syringe back into the syringe holder and click "Open fill port"

** Then connect the plastic tubing to the instrument syringe and clean it

Picture adopted from ⁴ VP-ITC MicroCalorimeter User's Manual, p. 17

by Gulden Camci-Unal

Cleaning the sample cell



Picture adopted from ⁴ VP-ITC MicroCalorimeter User's Manual, p. 47

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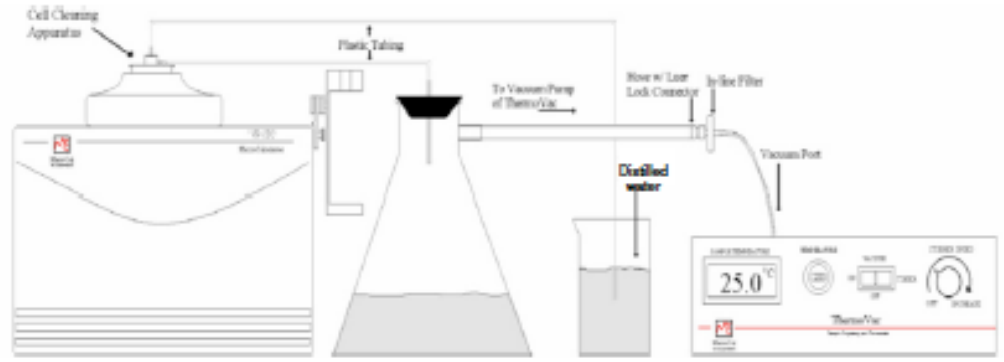
Cleaning the sample cell (Cont'd)



Picture adopted from [4] VP-ITC MicroCalorimeter User's Manual, p. 48

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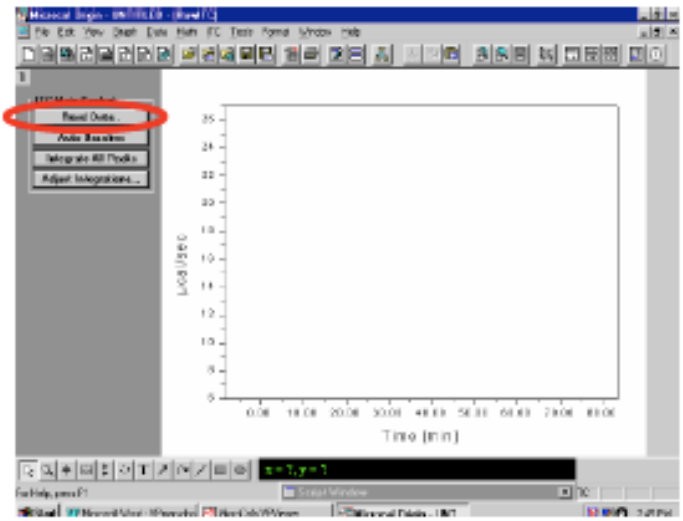
Data Analysis

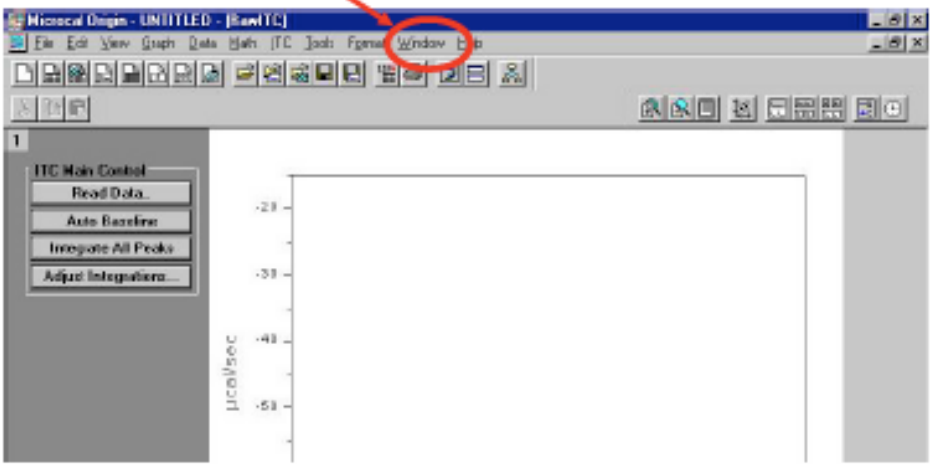
- 1- Open ORIGIN 7
- 2- Click "Read data", find your experiment file and say OPEN



Picture adopted from [8] ITC Data Analysis in Origin Tutorial Guide, p. 9

Data Analysis (Cont'd)

- 3- Under "Window" find "Raw ITC", click "Read data", find your *blank experiment* file and say "OPEN"



Picture adopted from ⁸ ITC Data Analysis in Origin Tutorial Guide, p. 9

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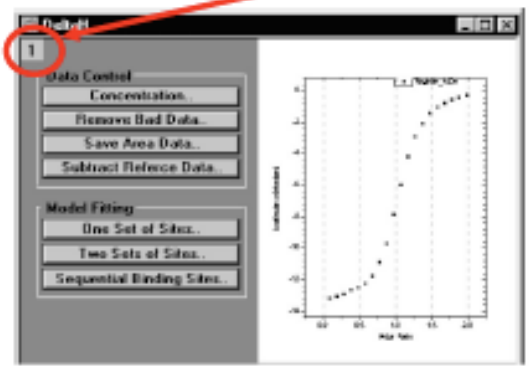
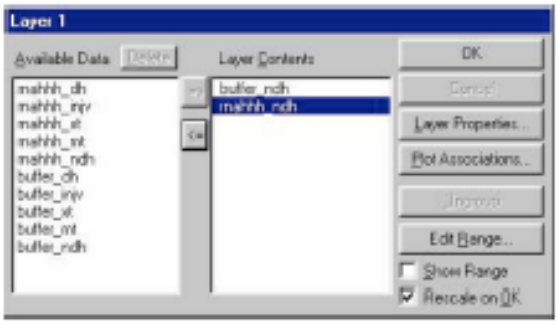
Data Analysis (Cont'd)

- 4- Double click [1], add your file name w/ extension _ndh and say OK

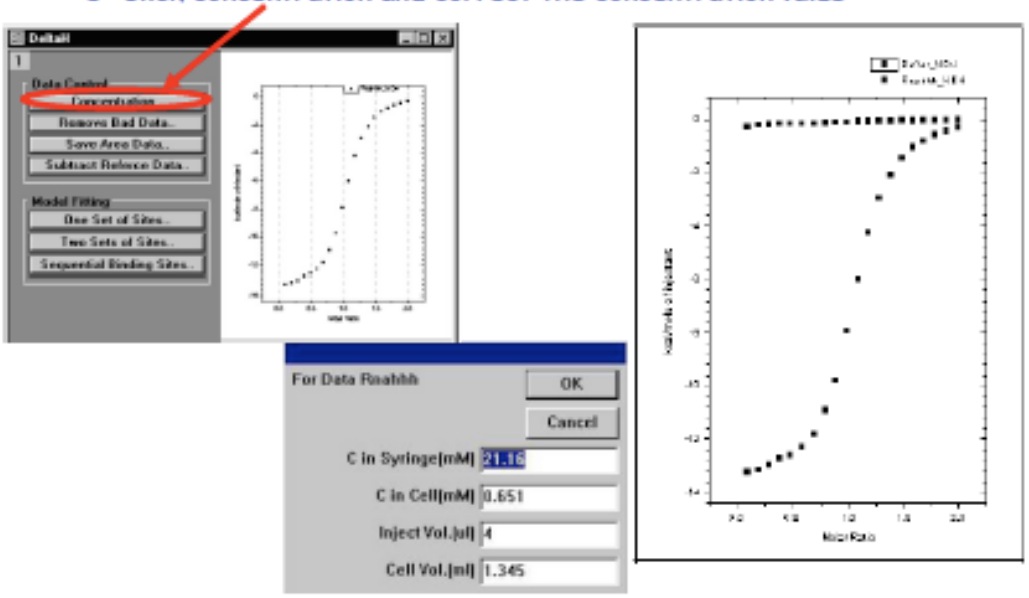




Pictures adopted from ⁸ ITC Data Analysis in Origin Tutorial Guide, p. 11and 32

Data Analysis (Cont'd)

- 5- Click concentration and correct the concentration value



Pictures adopted from " ITC Data Analysis in Origin Tutorial Guide, p. 11, 12 and 34

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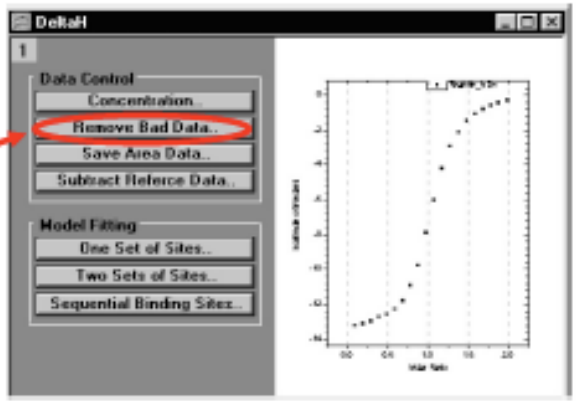
Data Analysis (Cont'd)

- 6- Subtract your "Reference data"

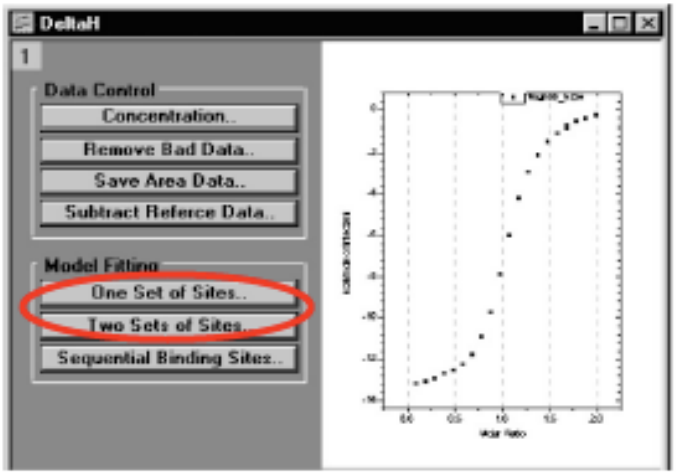
- 7- Remove bad data



Pictures adopted from " ITC Data Analysis in Origin Tutorial Guide, p. 11 and 34

Data Analysis (Cont'd)

- 8- Model fitting: pick "One set" or "Two sets of sites"



Picture adapted from ¹ ITC Data Analysis in Origin Tutorial Guide, p. 11

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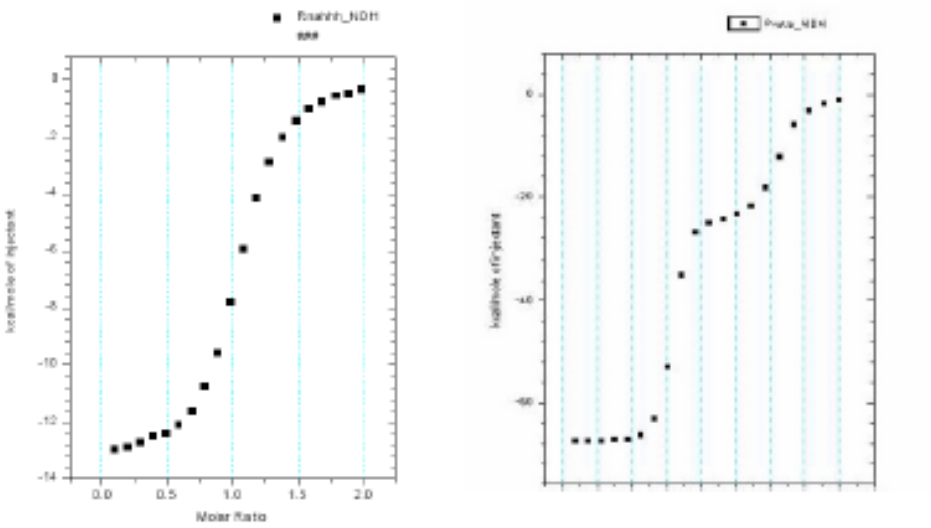
Data Analysis (Cont'd)

- One set of sites

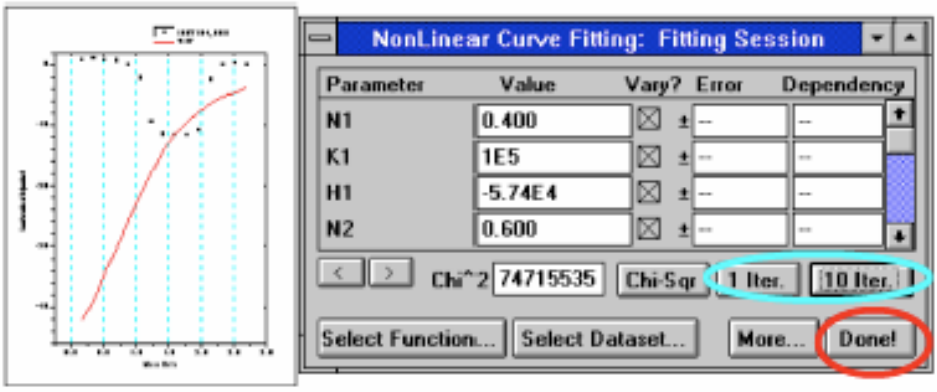
Two sets of sites



Figures adapted from ¹ ITC Data Analysis in Origin Tutorial Guide, p. 35 and 64

Data Analysis (Cont'd)

- 9- Do iterations
- 10- When "Chi-sqr is NOT reduced" anymore, click DONE



Picture adopted from online version of " ITC Data Analysis in Origin Tutorial Guide Version 5, Lesson 1
http://www.microcalorimetry.com/files/tech_docs2/itc_tutorial_origin_5.pdf

Data Analysis (Cont'd)

- 11- Under "ITC" click FINAL FIGURE

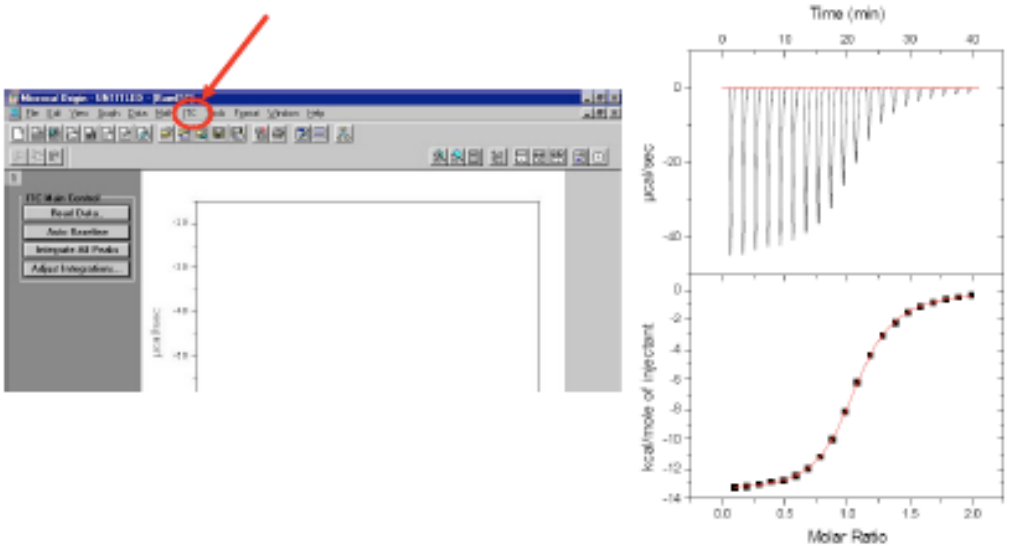


Figure adopted from " ITC Data Analysis in Origin Tutorial Guide, p. 17

Data Analysis (Cont'd)

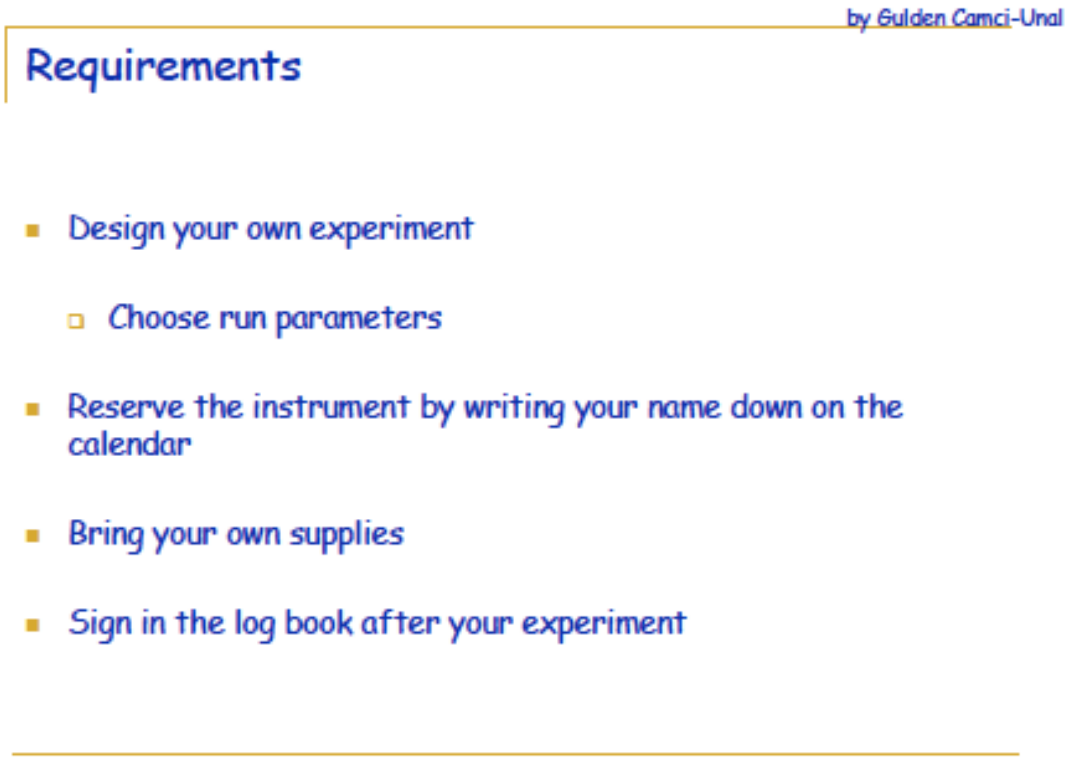
- 12- Save it as project file (*.OPJ)
- 13- When you are done w/ everything , click EXIT

Troubleshooting

- **ERROR MESSAGE: "No Injector_Response, Injector Disabled!"**
  - Cause: the power up sequence ! When starting the VP-ITC program, follow this specific sequence to initialize the injection system:
    - 1- Turn VP-ITC power switch ON
      - If the switch was already on, turn it off for couple of seconds, then turn it back ON
    - 2- Open the VPViewer application

    PROBLEM SOLVED!

[4] http://www.microcalorimetry.com/index.php?id=64



Reminders:

- Report all problems with the instrument to Steve. Make an entry in the logbook, but follow up with an e-mail to sveysey@iastate.edu.
- Handle syringes carefully; be careful not to lose the small stir bars.
- Updated versions of manuals, technical notes, et cetera can be found at our website www.cif.iastate.edu.
- You must properly complete an entry in the logbook each time you use the instrument.
- You may reserve up to 12 usage blocks per month without approval. Additional blocks must be approved by Steve.
- Scheduling mistakes should be neatly crossed out with one line so that the original name is still legible. Send an email to Steve explaining why you will not be using the time you have scheduled. You may be charged for scheduled but unused blocks.

APPENDIX A – VP-ITC TRAINING TUTORIALS

After reviewing Guldens training guide, you must successfully complete the three practical measurements described below. NOTE: You must also complete the Origin Data Processing – Lesson 1 available at our website. This will ensure that you have developed sufficient expertise to use the ITC properly without causing problems for other users.

Tutorial 1. Water to Water Baseline

1. Log on to the VPC-ITC control computer.

2. Check the logbook to see when the water in the reference cell was last replaced. If it has not been replaced within the past month, contact Steve and arrange to have the reference cell solution refreshed.

3. Degas approximately 20 mls of distilled water for at least five minutes. This degassed water will be the solution that will go into the sample cell and the syringe.

4. Load your 2.5 ml glass-filling syringe with water. Remove air bubbles. Insert the long needle into the sample cell entry tube (center tube) and carefully slide it down until it touches the bottom. Lift it up slightly and slowly depress the plunger to so that the cell fills from the bottom up. When you see the water level reach the top of trhe narrow access tube, depress the syringe plunger quickly 1-3 times to deliver abrupt bursts of about 0.25 ml. This will dislodge small bubbles that may have attached near the bottom of the cell.

5. Slowly withdraw the syringe until it is resting on the lip at the top of the sample cell entry tube. Carefully suck up any water that is in the funnel area just above the entry tube.

6. Launch the VPViewer application. <logon as your account>

7. Call up the water-water parameter set *<Load Run File; select water.inj; Open>*

8. Enter a filename for the water-water data you are about to acquire. No spaces, periods or hyphens; maximum of 16 characters.

9. Verify that the VP-ITC thermostat temperature is set to the desired run temperature of 25 degrees.

10. Load the ITC pipette. Place the water titrant into the pipette filling tube, then place the filling tube into the bottom of the pipette stand. Carefully insert the auto-pipette into the pipette stand. ***Be very careful not to hit the long needle of the injection syringe against any object. The needle must not be bent.***

11. In the software, select the ITC controls window. Click on the **Open Fill Port** button. The fine-thread screw controlling the syringe plunger should be observed to turn, and the plunger should move upwards until the white Teflon plunger tip is positioned just above the fill port.

12. Attach the tube of the **plastic filling syringe** to the **filling port of the injection syringe.**

13. Slowly withdraw the plunger of the plastic filling syringe to draw up the titrant solution until the solution begins to enter the filling syringe. Clicl on the Close Fill Port button. The injection syringe plunger tip should move to

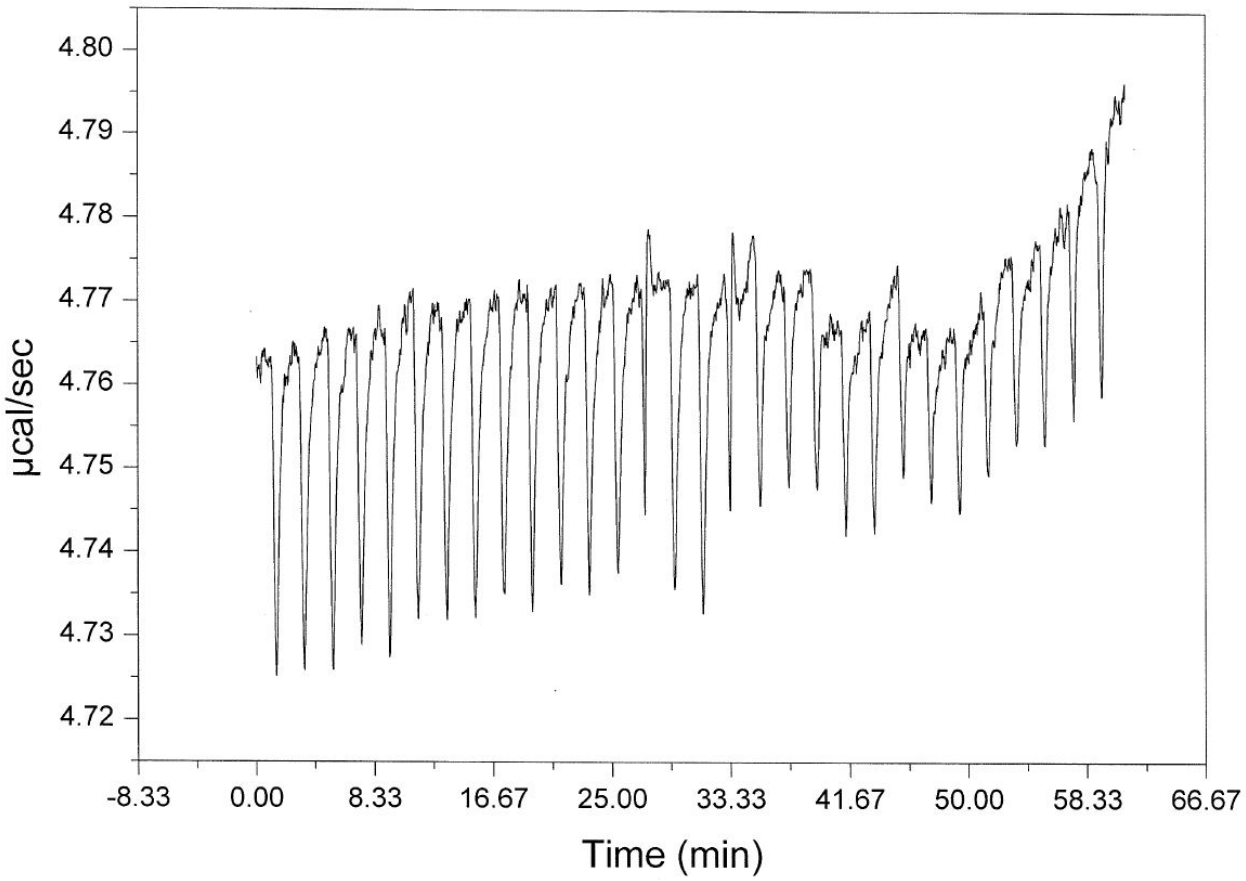
just below the filling port orifice. Remove the hose of the plastic filling syringe from the filling port of the injection syringe.

14. Click on the **Purge->Refill** button. The auto pipette screw will depress the plunger filling syringe, then draw it back up again. This will help dislodge air bubbles that may be in the syringe. Repeat the Purge->Refill process two more times.

15. Carefully remove the pipette from its stand by picking it straight up until the glass barrel of the injection syringe is above the top part of the pipette stand. Carefully move the pipette so that it is directly above the center-positioned sample cell access tube. Insert the pipette into the sample cell access tube by holding the pipette vertically and slowly lowering the pipette. When the pipette is almost completely inserted you may have to push down slightly to compensate for the resistance of the rubber o-ring so that the pipette is properly seated.

16. Select the START button to begin the experiment. You may receive a query about "*Invalid Cell or Syringe Concentration".* This is irrelevant for the water-water titration. Click **Yes** to continue.

The entire run will take approximately 30 minutes. The results should be similar to the graph shown below.



Tutorial 2. Methanol to Water Titration

A methanol injection run requires a higher level of precise preparation and operation of the VP-ITC instrument than the water-to-water- run.

Follow the procedures recommended in the water-to-water tutorial for preparation of solutions, degassing, filling the cell and injection syringe, et cetera.

Sample Preparation

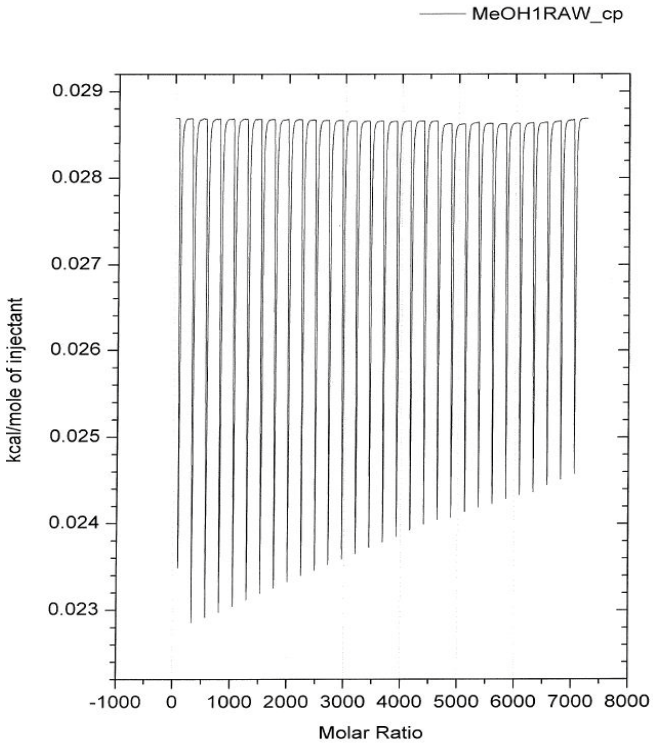
- Fill the sample cell with degassed water.
- Prepare 20 ml of degassed water in a glass vial and add 0.5 ml of methanol to make a 2.5% methanol solution.
- Degas the methanol solution (with stirring) for 5 minutes.
- Load the methanol solution into the injection syringe of he pipette.

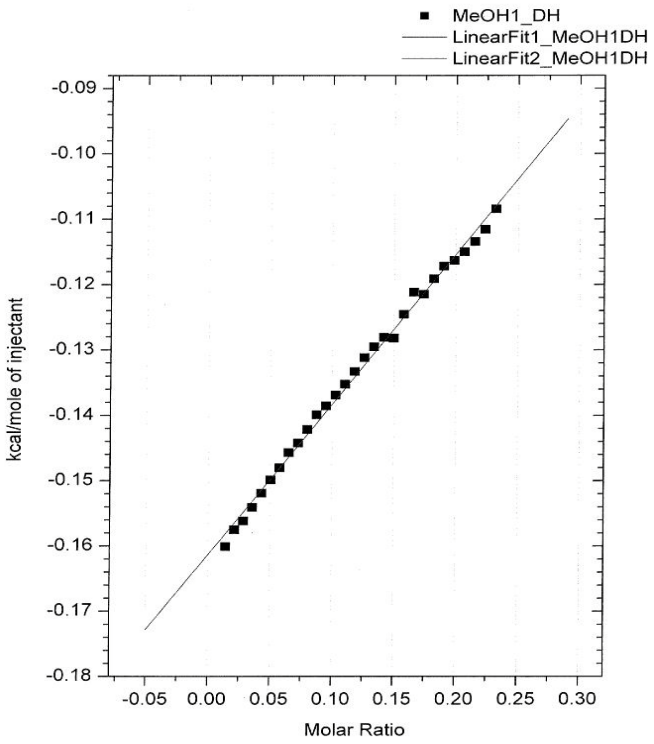
Conducting the Experiment

- Load the parameters from the methanol.inj file. <*Load Run File; select methanol.inj; Open*>
- Enter a filename for the methanol-water titration data.
- Execute the run and allow the system to complete the experiment.

Analyzing the Data

- After the run is complete, minimize VPViewer.
- Open the separate Origin 70 program for data analysis. Read in the data you have just acquired. The raw ITC data should look like the results in the figure below.
- View the data in the DeltaH window. Generate a linear regression fit; the first data point should be excluded. The DeltaH data should look like the results in the figure below.





Tutorial 3. EDTA – CaCl2 Titration

The final tutorial will require that you are familiar with the more advanced data processing techniques available in the Origin 7 software. Nine Origin Data Processing lessons are available at our website. You must complete Lesson 1 "Routine ITC Data Analysis and Fitting" before you will be able to complete this tutorial.

Sample Preparation. Obtain a test kit labeled "EDTA – CaCl2 Titration" from the refrigerator. Each kit will contain

- A tube of EDTA solution labeled with concentration and buffer conditions
- A tube of CaCl2 solution labeled with concentration and buffer conditions
- A vial of 10 mM MES buffer solution for rinsing the cell prior to sample loading

IMPORTANT NOTE: The control titrations for this experiment will not be performed. The system will be going to saturation and the small, repeatable peaks at the end of a well designed experiment are a very good representation of control heats.

Setting Up the Experiment. Load the parameters from the edta.inj file. <*Load Run File; select edta.inj; Open*>

Note: The instrument should be thermostatted at 25^{o}C to facilitate a faster start.

Total # Injections	29	Volume 1st Injection	2
Cell Temperature	25	Duration 1st Injection	4
Reference Power	10	Volume after 1st Injection	10
Initial Delay	60	Duration after 1st Injection	20
Syringe Concentration	1	Injection Spacing	210
Cell Concentration	0.1	Filter Period	2
Stir Speed	307	Feedback Mode/Gain	High
		ITC Equilibration Options	Fast Equil; Auto

NOTE: Cell and syringe solution concentrations need to be entered manually in all cases.

Loading Samples

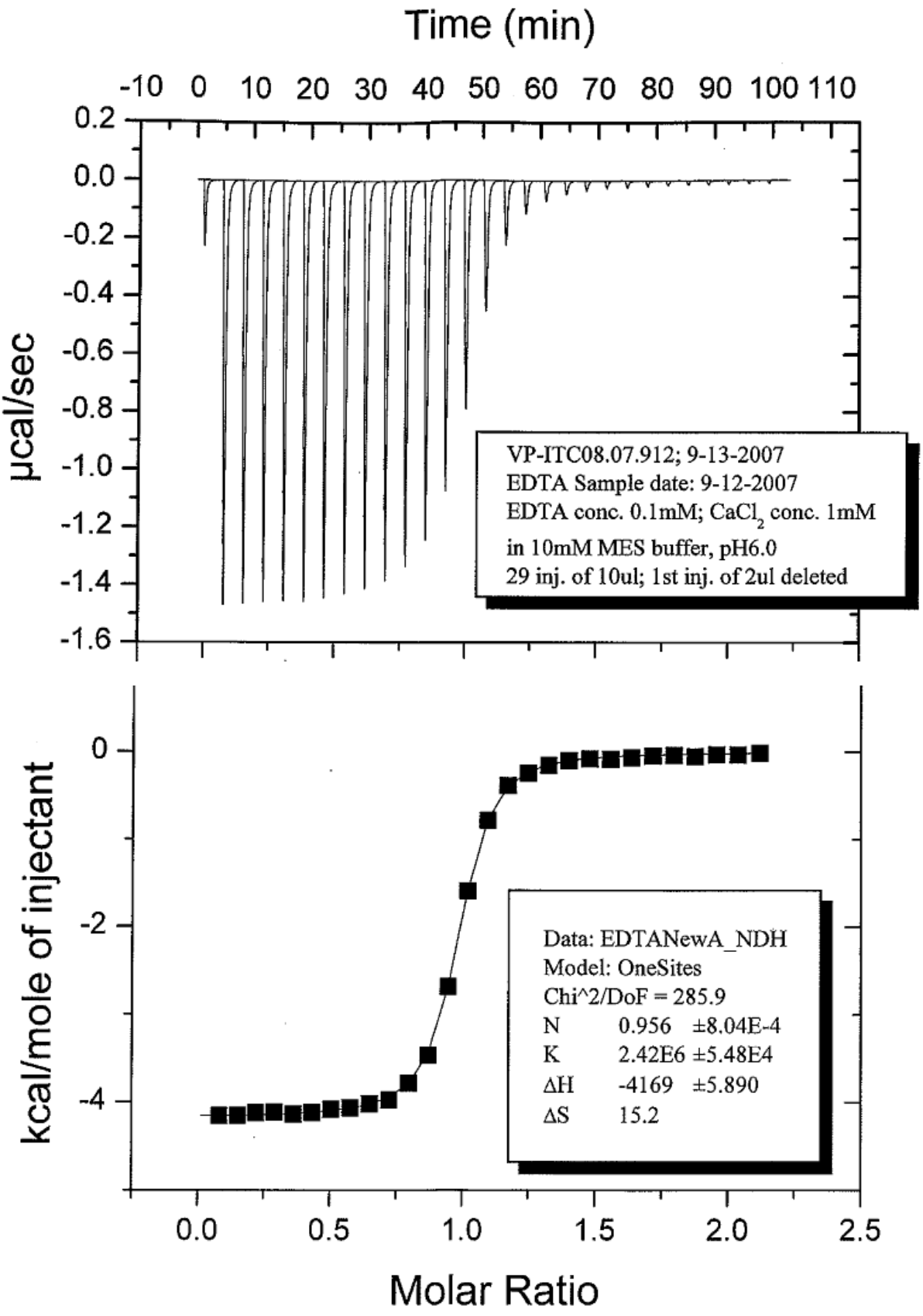
1. Rinse the sample cell with 10mM MES buffer two times
2. De-gas both the EDTA as well as the $CaCl_2$ samples for 5 minutes only
3. Load 2ml of 0.1mM EDTA into sample cell; pipette the liquid up and down for several times and soak for at least 2 minutes
4. Empty the sample cell, and reload fresh EDTA for the experiment
5. Clean the injection syringe with methanol and dry thoroughly before loading 1.0mM $CaCl_2$

Analyze the Data

Analyze the data in Origin and compare the results to those on the graph below. If the results are within the specifications stated below, the instrument (and the operator) is performing correctly and you are ready to run experimental samples.

The results which you obtain should be very close (i.e. N value +/- 5%; K value +/- 20 %; and Delta H value +/- 10%) to the values shown in the results at the end of this tutorial

Expected Experimental Results



It should be noted that no control heats were subtracted during the analysis of the attached results and that the same procedure should be applied to your results. It should also be noted that the experimental energies for the peaks were obtained using the automated integrations within Origin, but that in some cases those automated integrations might need to be manually adjusted to get agreement of the results.

Common Problems – What to Do?

If your results do not agree with the expected results then you might want to repeat the experiment to see if your results are reproducible, or not. In repeating the experiment, consider the following possible causes of bad results:

1.) Sample dilution will affect your results and specifically will change the resulting 'N' value obtained from the fit of your binding isotherm. Dilution of the cell solution (EDTA) will lower the 'N' value and dilution of the titrant solution ($CaCl_2$) will increase the 'N' value. The net change in 'N' will be the difference in dilutions between the cell and titrant solutions. **Avoid dilution factor** by using completely clean and dry transfer syringes, and also by pre-soaking the cell with EDTA.
2.) Sample contamination from the MeOH can cause for bad experimental results. Be sure to thoroughly dry the MeOH from the injection syringe prior to loading it with $CaCl_2$.
3.) A dirty sample cell or a dirty injection syringe can affect your results and also the quality of the data. Be sure to thoroughly clean the sample cell and injection syringe prior to carrying out the experiment with the standard sample kit.
4.) If problems persist then e-mail labcrew-us@ge.com and specify your instrument model and be sure to attach the raw data files that you generated (*.itc files). We will review your results and provide you with an assessment as well as any pertinent suggestions for improvement.

APPENDIX B ORIGIN 7 LESSONS

You must complete Lesson 1 before attempting to use the VP-ITC. THe raw data files associated with the lessons can be found at:
All of the lessons are posted as PDF files at our website, www.cif.iastate.edu.

Lesson 1	Routine ITC Data Analysis and Fitting
Lesson 2	Setting Baseline and Integration Range
Lesson 3	Deleting bad data
Lesson 4	Analyzing Multiple Runs and Subtracting Reference
Lesson 5	ITC Data Handling
Lesson 6	Modifying Templates
Lesson 7	Advanced curve Fitting
Lesson 8	Autosampler Data (optional accessory – we do not have it)
Lesson 9	Other Useful Details
Lesson 10	Equations Used for Fitting ITC Data