Isothermal titration calorimetry

S2004 – METHODS FOR CHARACTERIZATION OF BIOMOLECULAR INTERACTIONS: CLASSICAL VERSUS MODERN

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Outline:

- Historical background
- Theory
- Study of interactions
- Instrumentation
- ITC data analysis

History of calorimetry

- Calorimetry – Latin *calor* – heat, Greek *μέτρον (-metry)* – to measure
  - thermodynamic technique based on measurement of heat that may be generated (exothermic process) or consumed (endothermic process) by sample

- Calorimeter – instrument for measuring the quantity of heat released or absorbed in process of chemical reaction

Calorimetric units

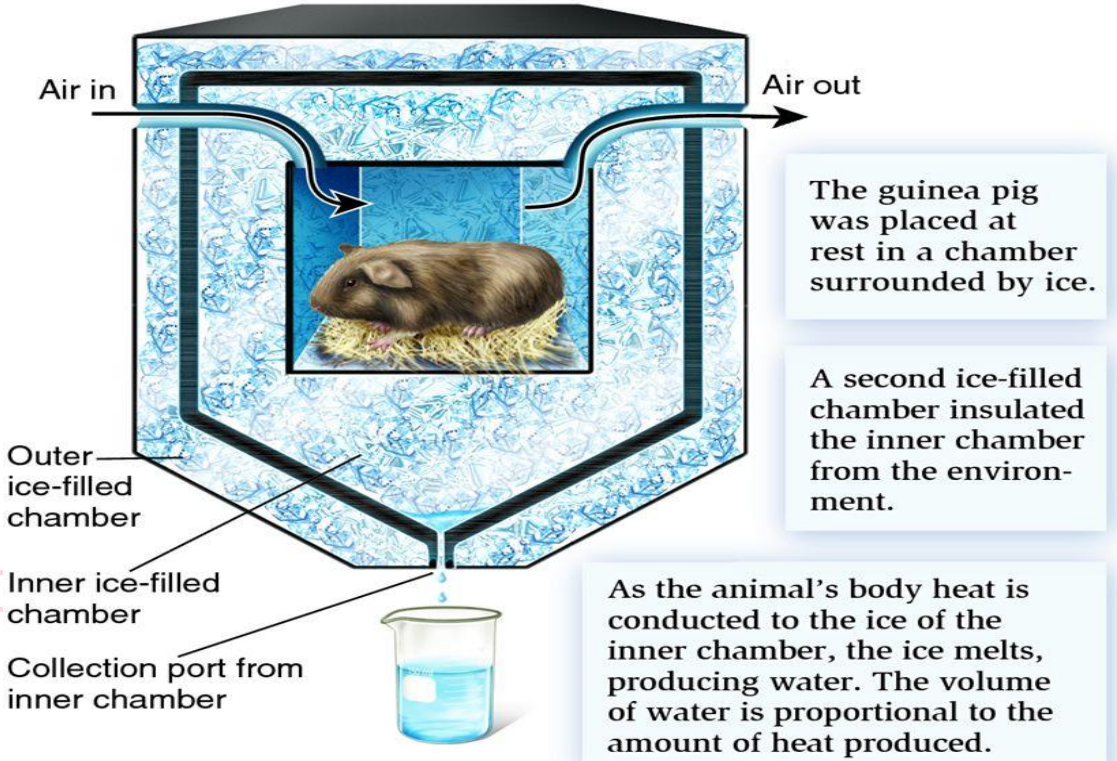
- A single calorie is the amount of energy required to increase the temperature of 1g of water by 1°C.
- A single joule is the amount of energy required to apply a force of 1 Newton over one meter of distance.

- 1 calorie = 4.184 J
  1 Calorie= 1 kcal = 4184 J
  1 J = 0.000239 kcal = 0.2390 cal

History of calorimetry: „Founding Fathers“

- **Joseph Black (1728 – 1799)**
  - „founder of the calorimetry“
  - first scientist who recognized the distinction between heat and temperature
- **Antoine Lavoisier (1743-1794)**
- **Pierre- Simon Laplace (1749-1827)**

First calorimeter - small guiney pig inside



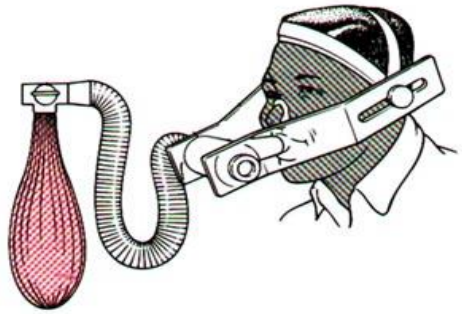
Calorimetry

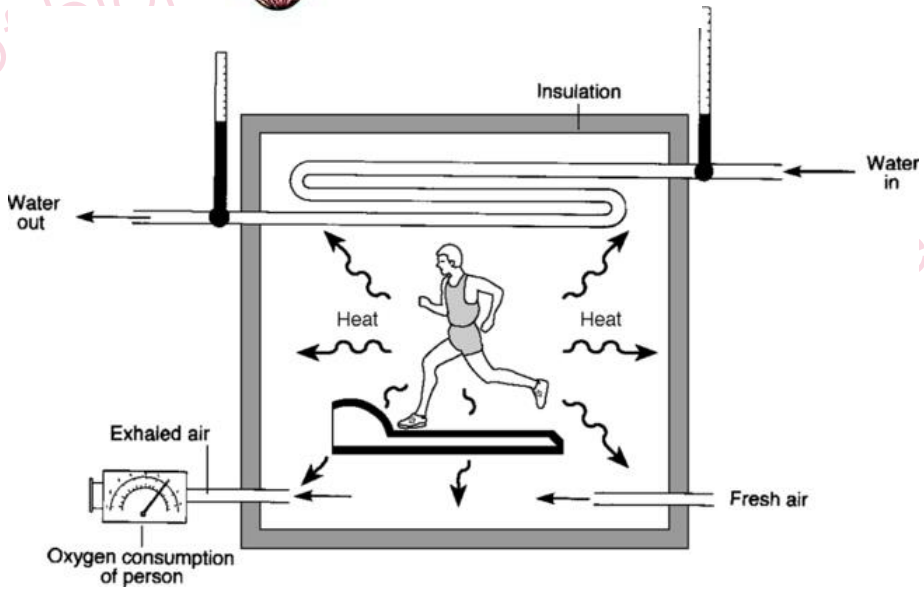
- **INDIRECT CALORIMETRY –**

  calculates the heat generated by living organism when their metabolic processes yield waste carbon dioxide

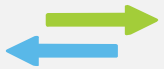
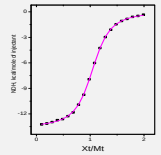
- **DIRECT CALORIMETRY –**

  measures heat generated by living organism by placing the entire organism inside the calorimeter for the measurement



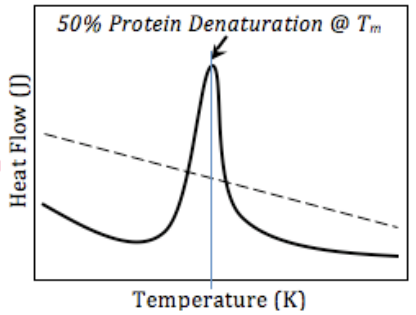


Microcalorimetry in cube:

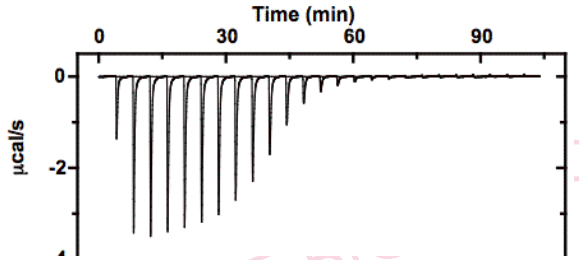
Microcalorimetry	Broad dynamic range	Information rich	Ease-of-use
• Direct measurement of heat change (ITC) • Direct measurement of melting transition temperature to predict thermal stability (DSC) 	• Native molecules in solution (biological relevance) • Very sensitive to accomodate range of affinities 	• All binding parameters (affinity, stoichiometry, enthalphy and entropy) in a single ITC experiment 	• No labeling or immobilization necessary • Wide range of solvent/buffer conditions 

Microcalorimetry

- **Differential scanning calorimetry DSC**
  - Biomolecular stability in solution
  - Provides insights into mechanisms of unfolding and refolding
  - Midpoint (Tm) determination



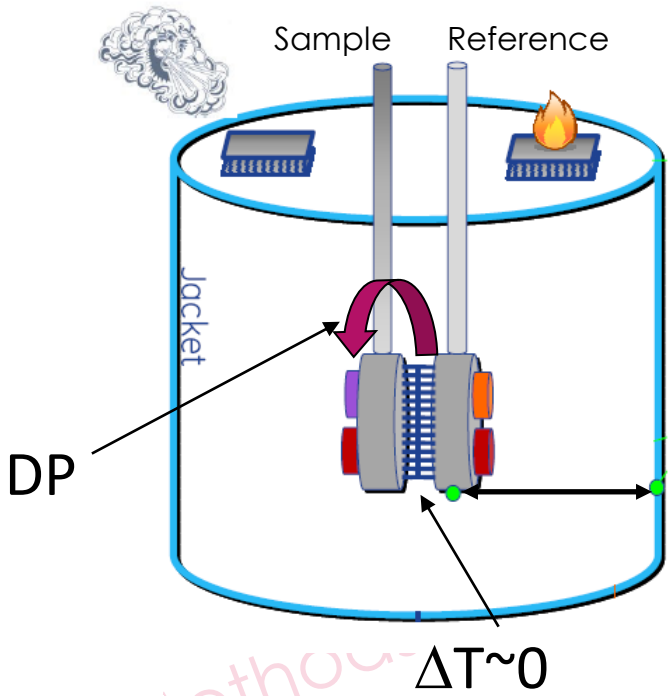
- **Isothermal titration calorimetry ITC**
  - Heat is released or absorbed as a result of the redistribution and formation of non- covalent bonds when the interacting molecules go from the free to the bound state.



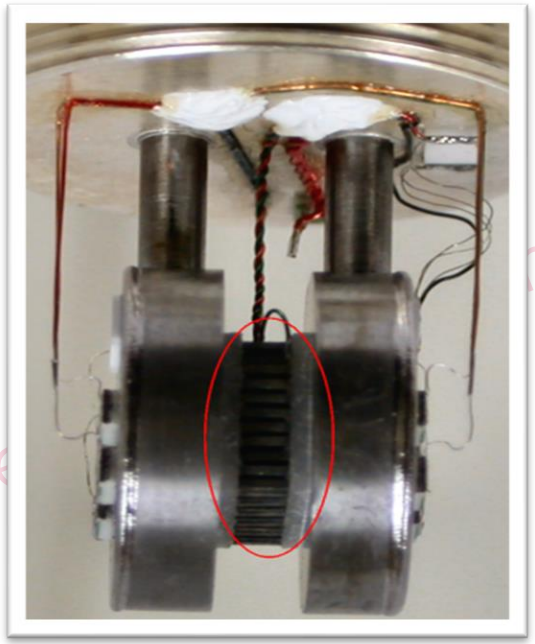
With isothermal titration calorimetry you can…

- Get quick K_Ds
- Measure target activity (Stoichiometry, active concentration)
- Protein batch activity comparison
- Confirm drug binding to target
- Use thermodynamics to guide lead optimization
- Measure enzyme kinetics

How does it work?



Reference Calibration Heater
Sample Calibration Heater
Cell Main Heater

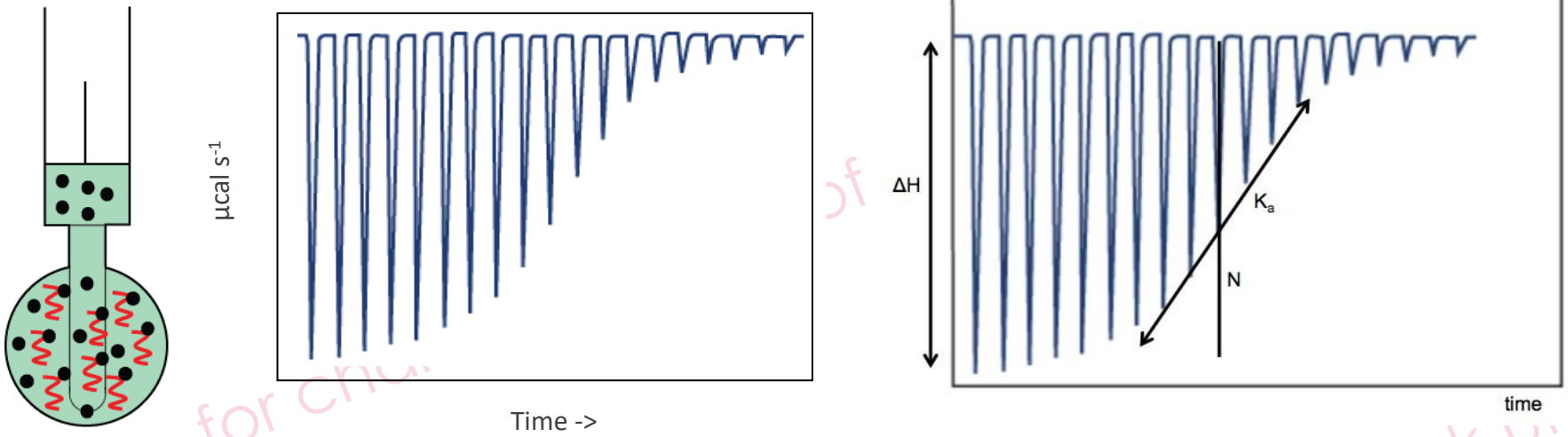


DP = Differential power
ΔT = Temperature difference

The DP is a measured power differential between the reference and sample cells to maintain a zero temperature between the cells

Basics of ITC experiment

Universal technique based on heat detection



Integration of heats are used to extract affinity (K_D), stoichiometry (N) and binding enthalpy (ΔH) using appropriate binding model

The energetics

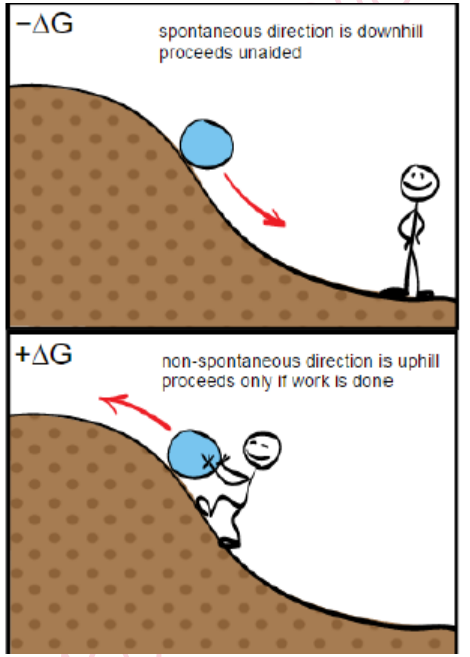
$$\Delta G = RT \ln K_D$$

$$\Delta G = \Delta H - T\Delta S$$

ΔH, **enthalpy** is indication of changes in hydrogen and van der Waals bonding

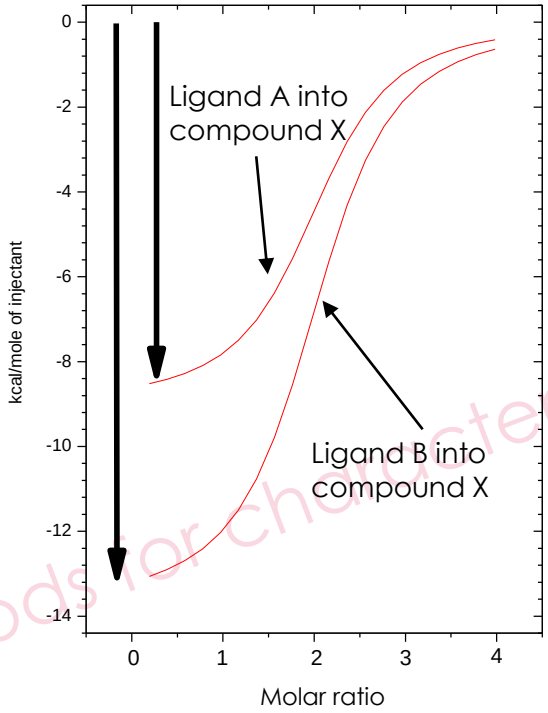
$-T\Delta S$, **entropy** is indication of changes in hydrophobic interaction and/or comformational changes

ΔG = Gibbs free energy
ΔH = Enthalpy
ΔS = Entropy
R = Gas constant = 1.985 cal K^{-1} mol^{-1}
T = Temperature in Kelvin = 273.15 + t ^0C
K_D = Affinity



The energetics

- The same affinity and stoichiometry but different enthalpy
- This tells us there are different binding mechanisms

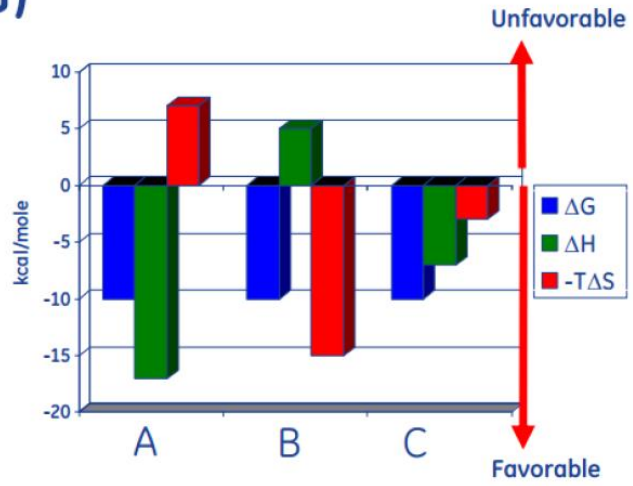


Same affinity, different energetics! All three interactions have the same binding energy (ΔG)

A. Good hydrogen bonding with unfavorable conformational change

B. Binding dominated by hydrophobic interaction

C. Favorable hydrogen bonds and hydrophobic interaction



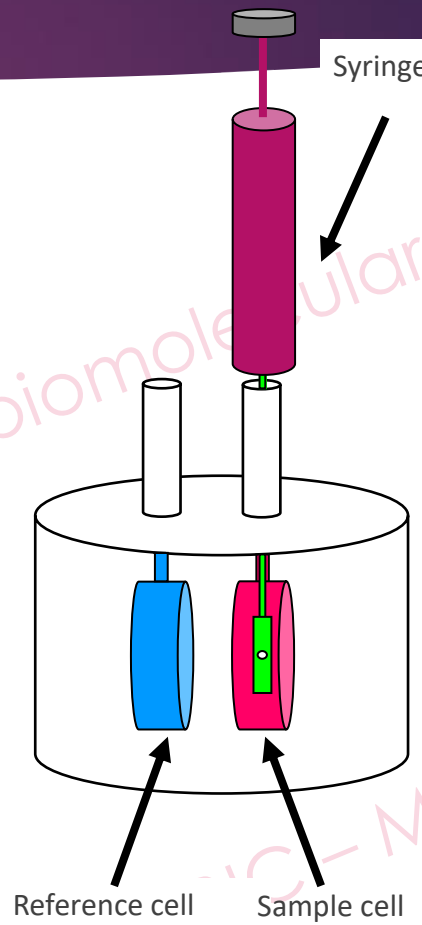
ITC results are used to get insights into mechanism of binding

ITC experiment

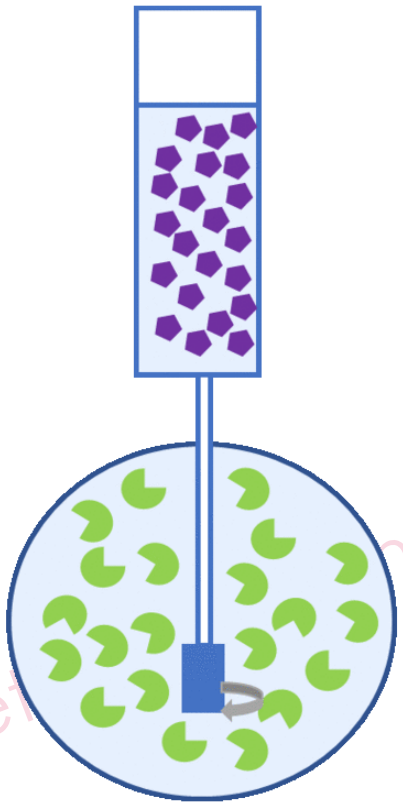
Standard set-up:

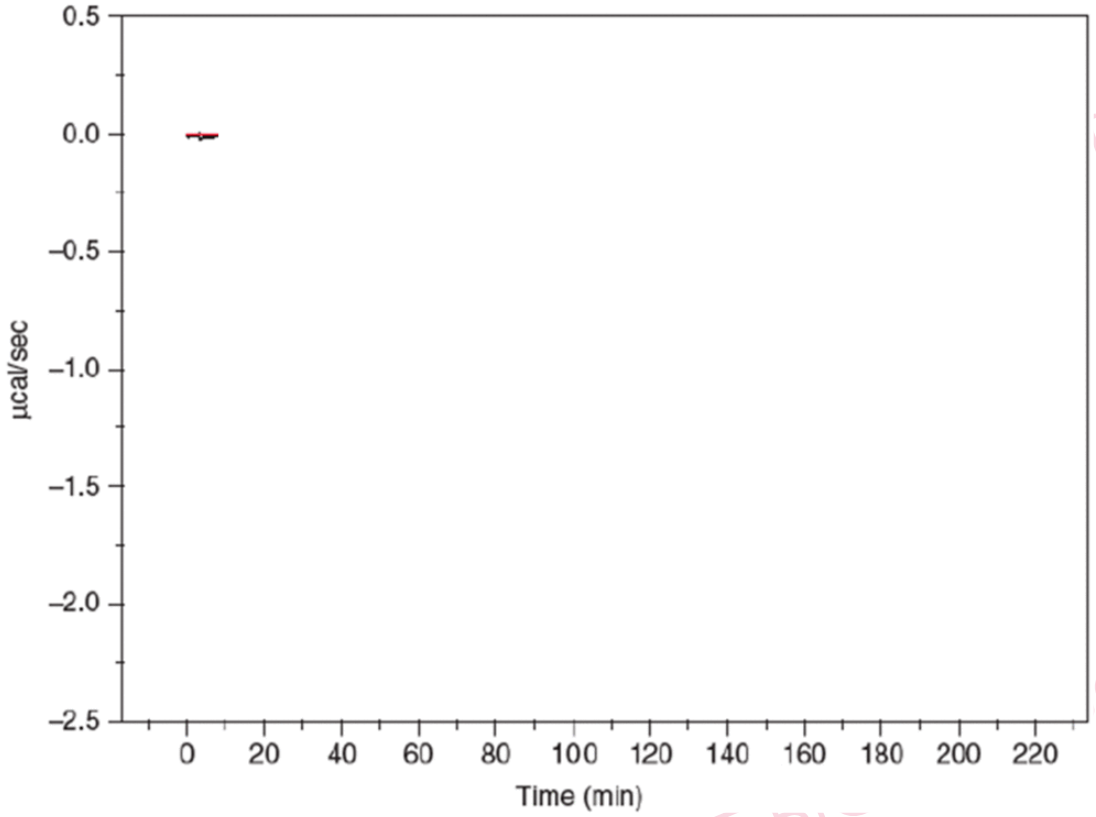
- "Ligand" in syringe
- "Macromolecule" in sample cell

- Reverse arrangement possible
- Concentration and other parameters necessary to set-up the experiment



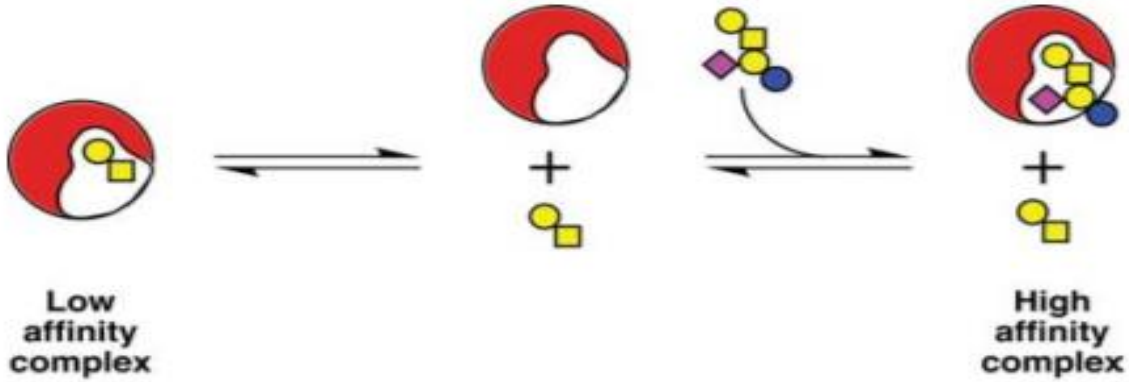
Performing an ITC experiment





Competition titration:

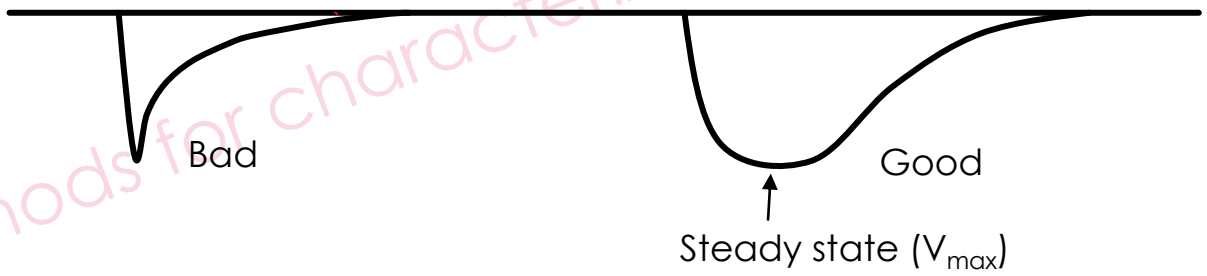
Very high and very low affinity systems can be studied using competition titrations



- High affinity ligand added to a solution of the low affinity complex
- High affinity ligand displaces the low affinity ligand
- Change in the apparent affinity and apparent enthalpy
- If parameters for one ligand are known, possible to calculate for the other ligand

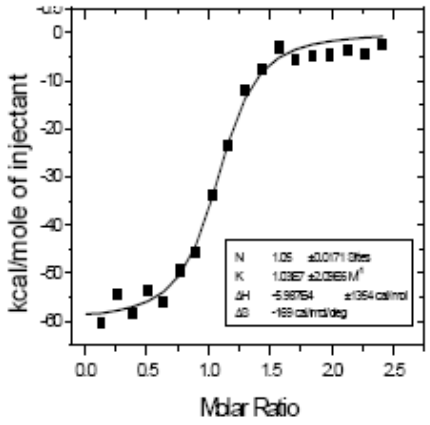
Single injection method

- This setup consist of one injection of substrate into enzyme with the aim to reach Vmax as fast as possible and observe the signal decay associated with substrate depletion and product formation.



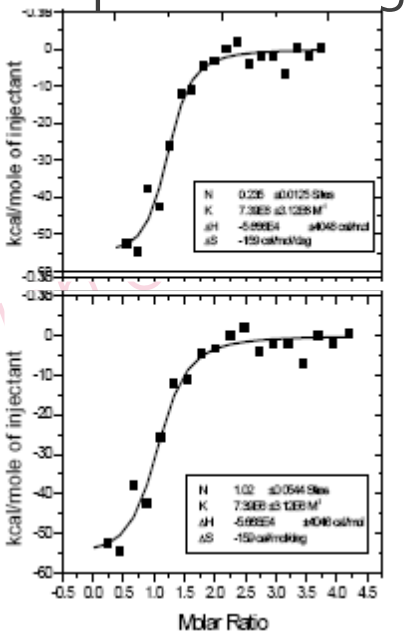
Assessment of protein quality by MicroCal™ iTC_{200} system

Peptide binding to protein Batch #1



N = 1.05
K_D = 97 nM

Peptide binding to protein Batch #2



50 µM Peptide
10 µM Protein X
N = 0.235
K_D = 135 nM

Re-analyzed as
2.3 µM Protein X
N = 1.02
K_D = 135 nM

- **100%** of Batch #1 protein active based on stoichiometry
- **23%** of Batch #2 protein active based on stoichiometry

Presented by L.Gao (Hoffmann-La Roche), poster at SBS 2009

Sample preparation

Sample preparation – „c value“

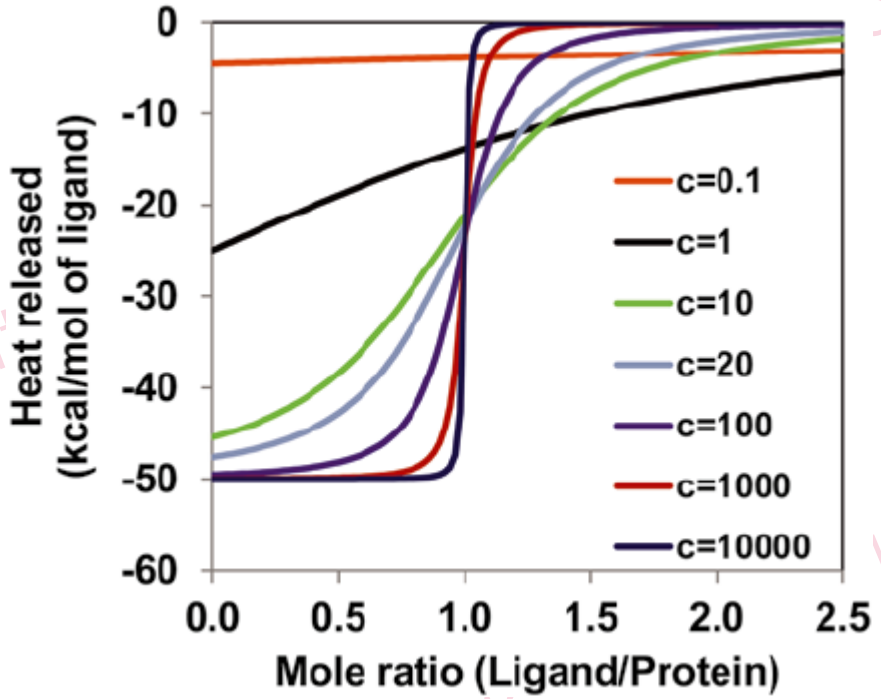
$C = [Protein]/K_D$

C = 10-100 Great

C = 5-10 and 100-500 Good

C = 1-5 and 500-1000 Accuracy limited

C = < 1 and > 1000 Competittive ITC experiment



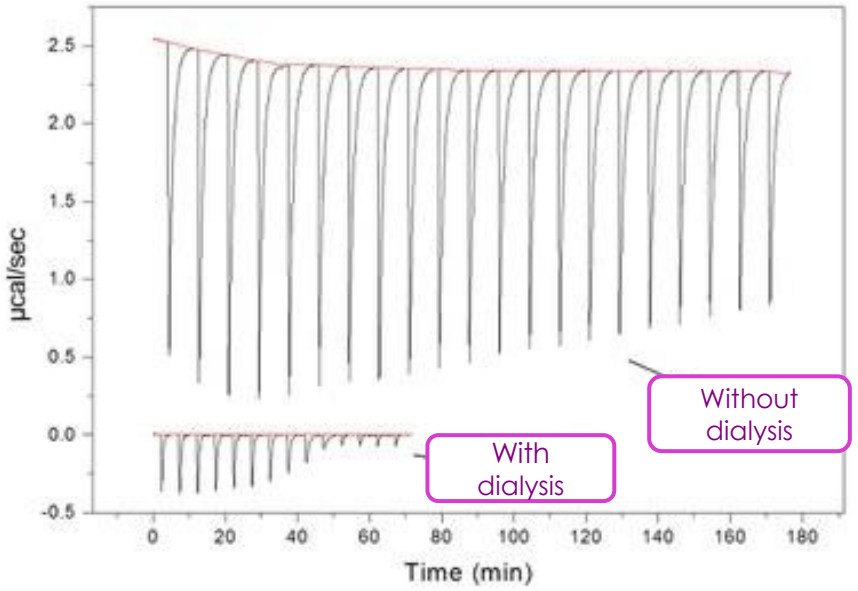
Sample preparation

- Stability of interacting molecules in conditions of the ITC experiment
- The cell and syringe buffers must be carefully matched. This is best accomplished by dialyzing both the macromolecule and the ligand in the same buffer.
- If the ligand is too small for dialysis then dialyze the macromolecule and then dissolve the ligand in the dialyze buffer
- Accurately measure protein concentration using A280nm

Poor sample preparation = poor data

- The data show possible difference of measurement of the sample before and after dialysis

- The large peaks were due to differences in the NaCl concentration between buffers



Instrumentation

Malvern Instruments

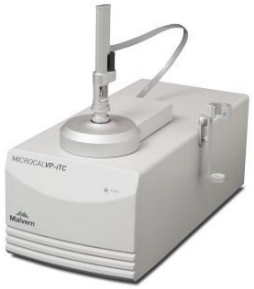
- Differential scanning calorimetry

MicroCal™ VP-DSC

MicroCal VP-Capillary DSC

- Isothermal titration calorimetry

MicroCal VP-ITC



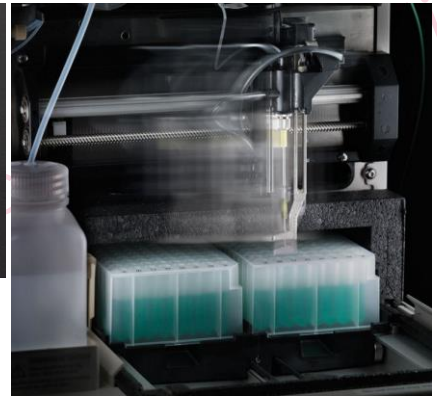
MicroCal iTC$_{200}$

MicroCal PEAQ™ ITC

MicroCal PEAQ ITC Automated

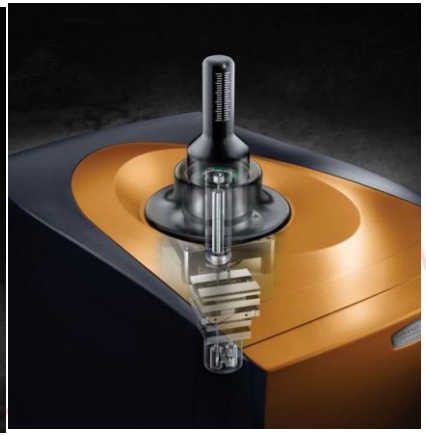
TA Instruments

- Differential scanning calorimetry



- Isothermal titration calorimetry

	Standard Volume	Low Volume
Cell Geometry	Fixed Cylindrical	Fixed Cylindrical
Cell Composition	24K Gold / Hastelloy	24K Gold
Active Cell Volume	1.0 mL	190 µL

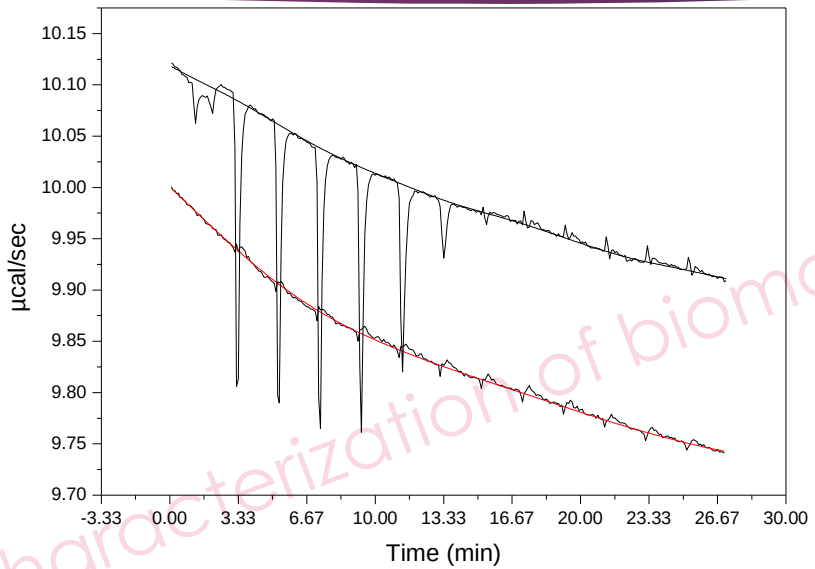


ITC – theory of data analysis

What we are going to discuss?

- Analysis of raw ITC data
- Data fitting
  - choice of model
  - fitting procedure
  - assessment of goodness of fit
- Global fit: fitting of multiple data sets

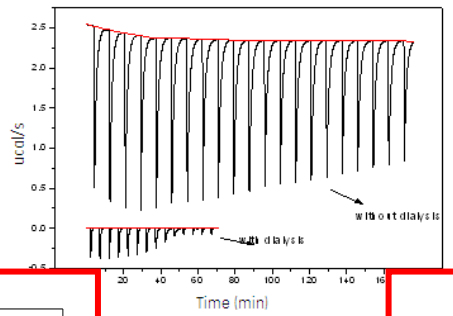
Raw ITC data

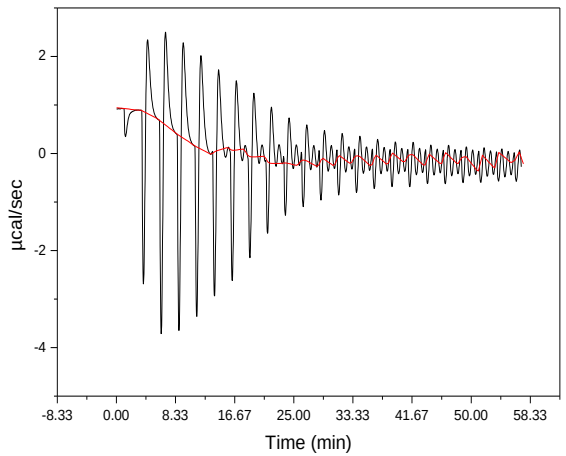


✓ **Make a sanity check of the raw data**

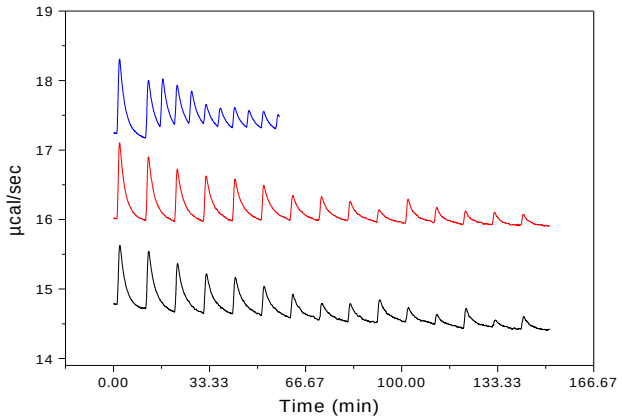
Bad quality raw ITC data

Buffer mismatch – no dialysis





Too low reference power

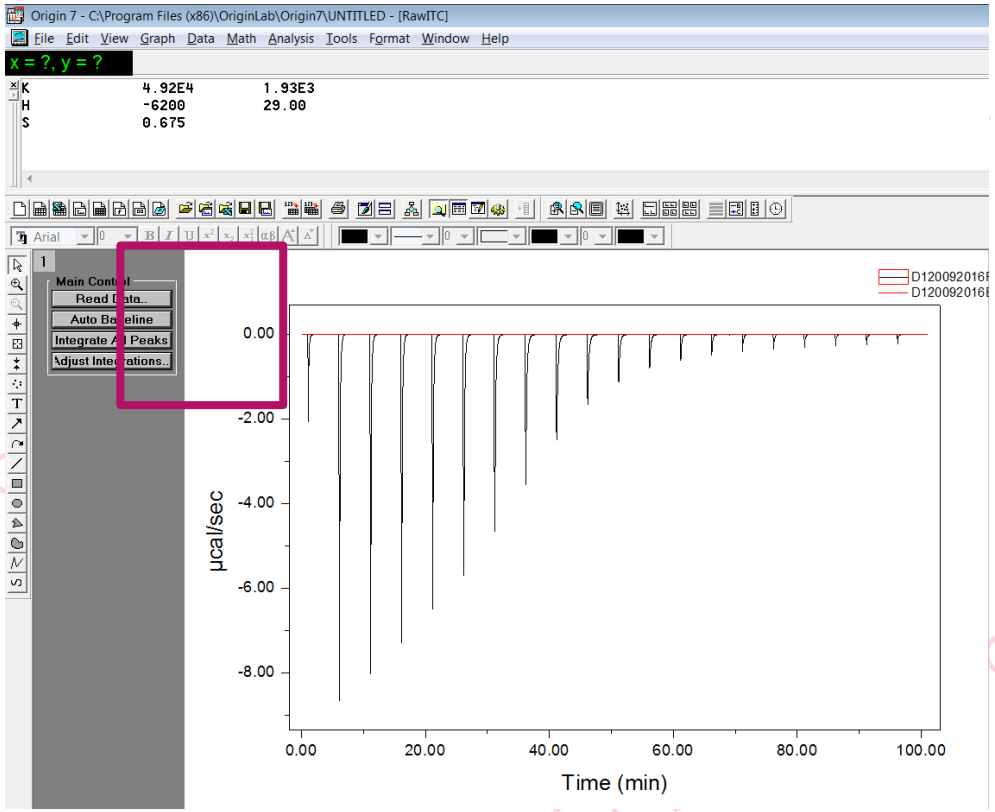


Too short spacing between the injections

Origin-based ITC data analysis software

First steps

- Adjust integrations
- Check concentrations
- Subtract control heats

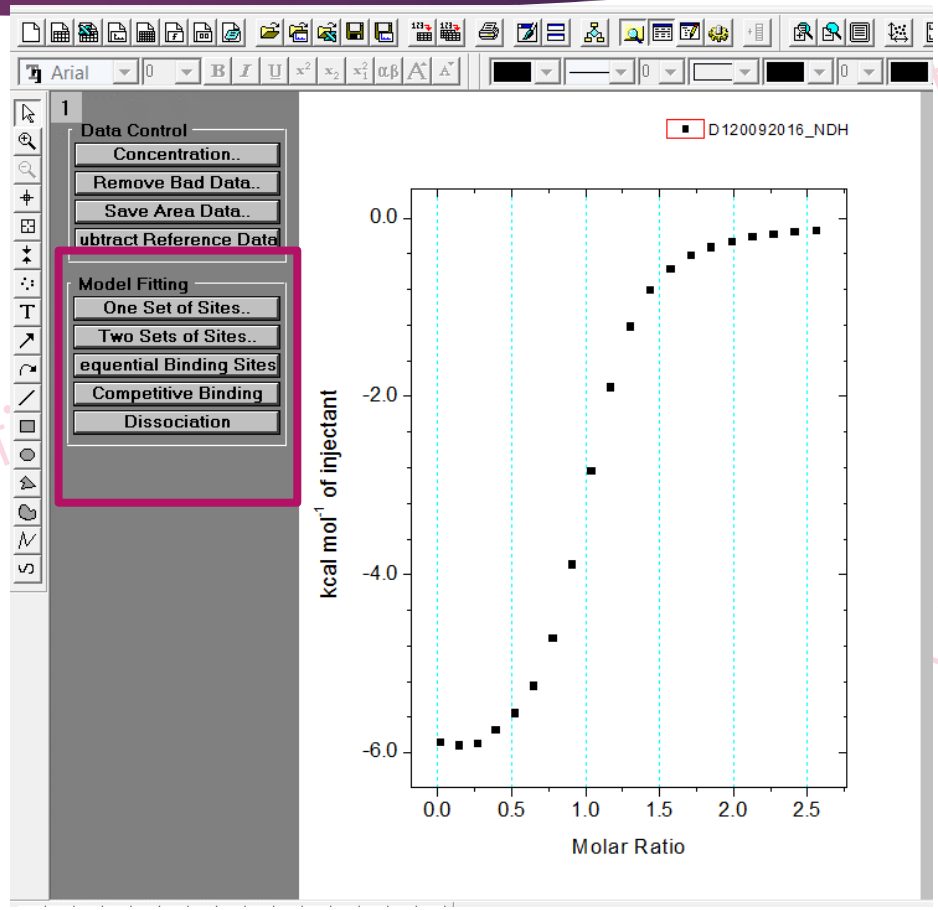


Choice of model

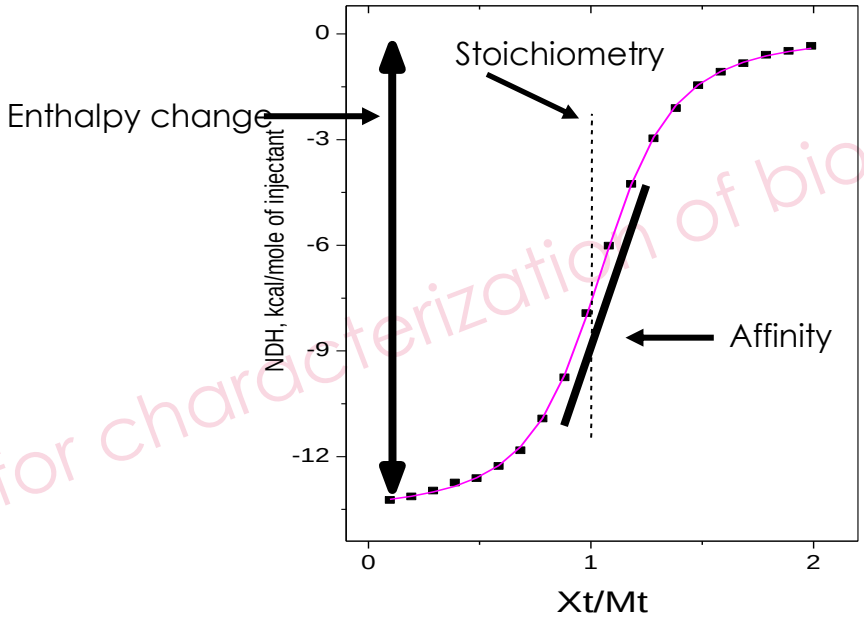
- Models supported by Origin:

- **4 binding models**
  - one set of sites
  - two sets of sites
  - sequential binding
  - competitive binding

- **a dimer dissiociation model**

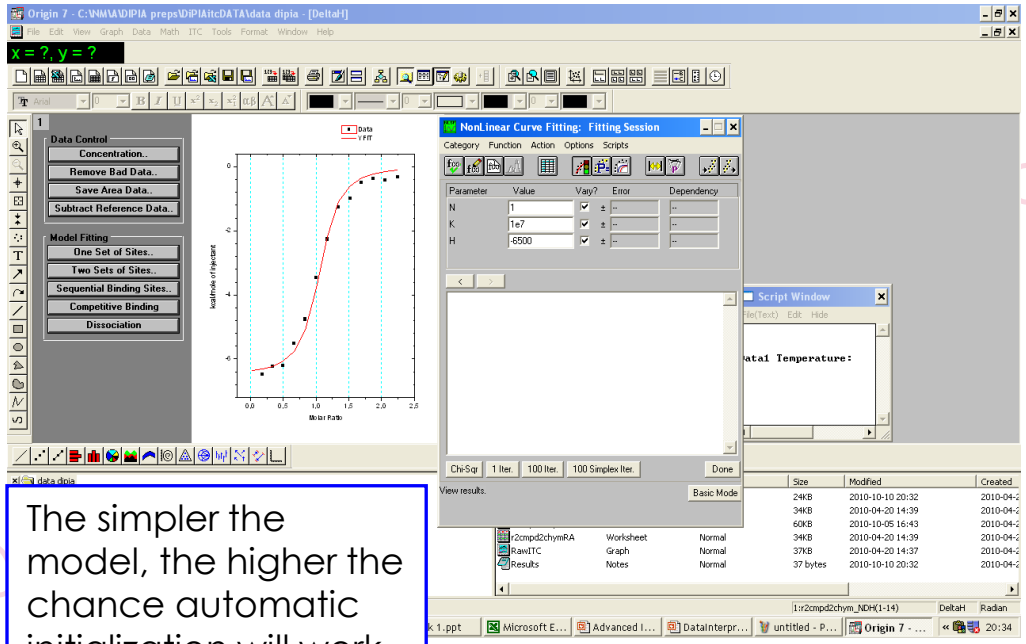


"One set of sites" model: parameters defined by binding isotherm

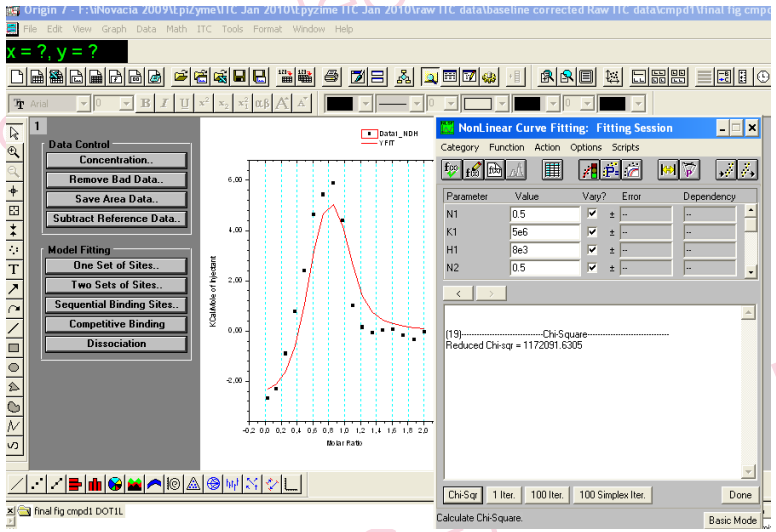


Manual fit initialization : your educated guess

If automatic initialization is not satifactory, in NL curve fitting box insert your "best-guess" values for parameters and click Chi-Sqr button. A simulated curve will appear next to your experimental data curve. Compare and decide.



The simpler the model, the higher the chance automatic initialization will work.

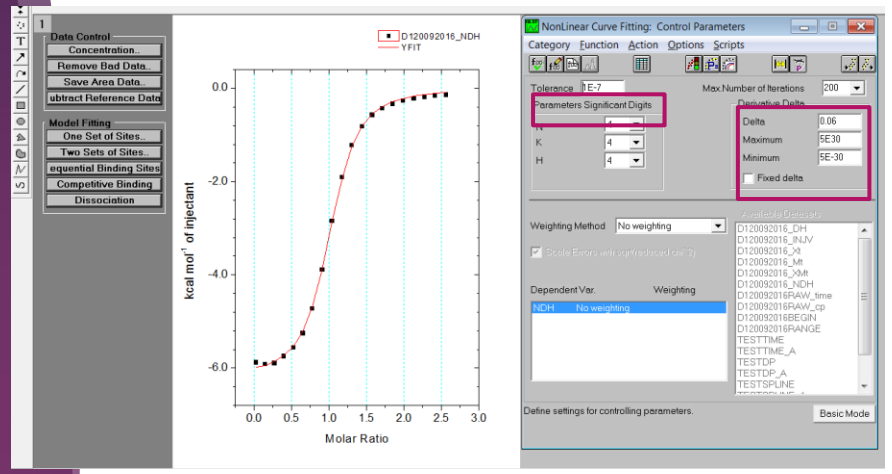


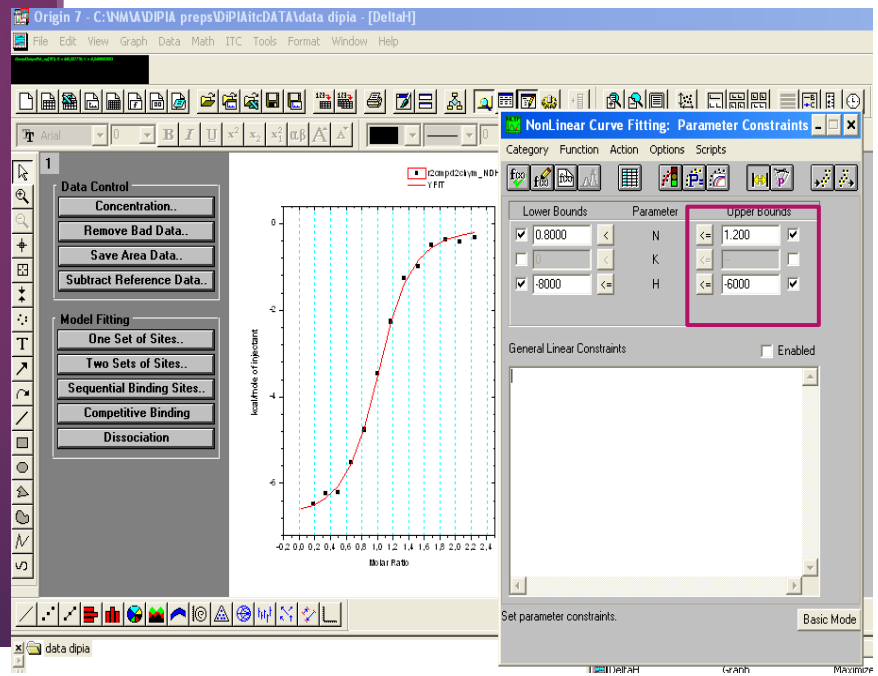
Control of fitting procedure

Tolerance-compare chi-sqr values between two successive iterations

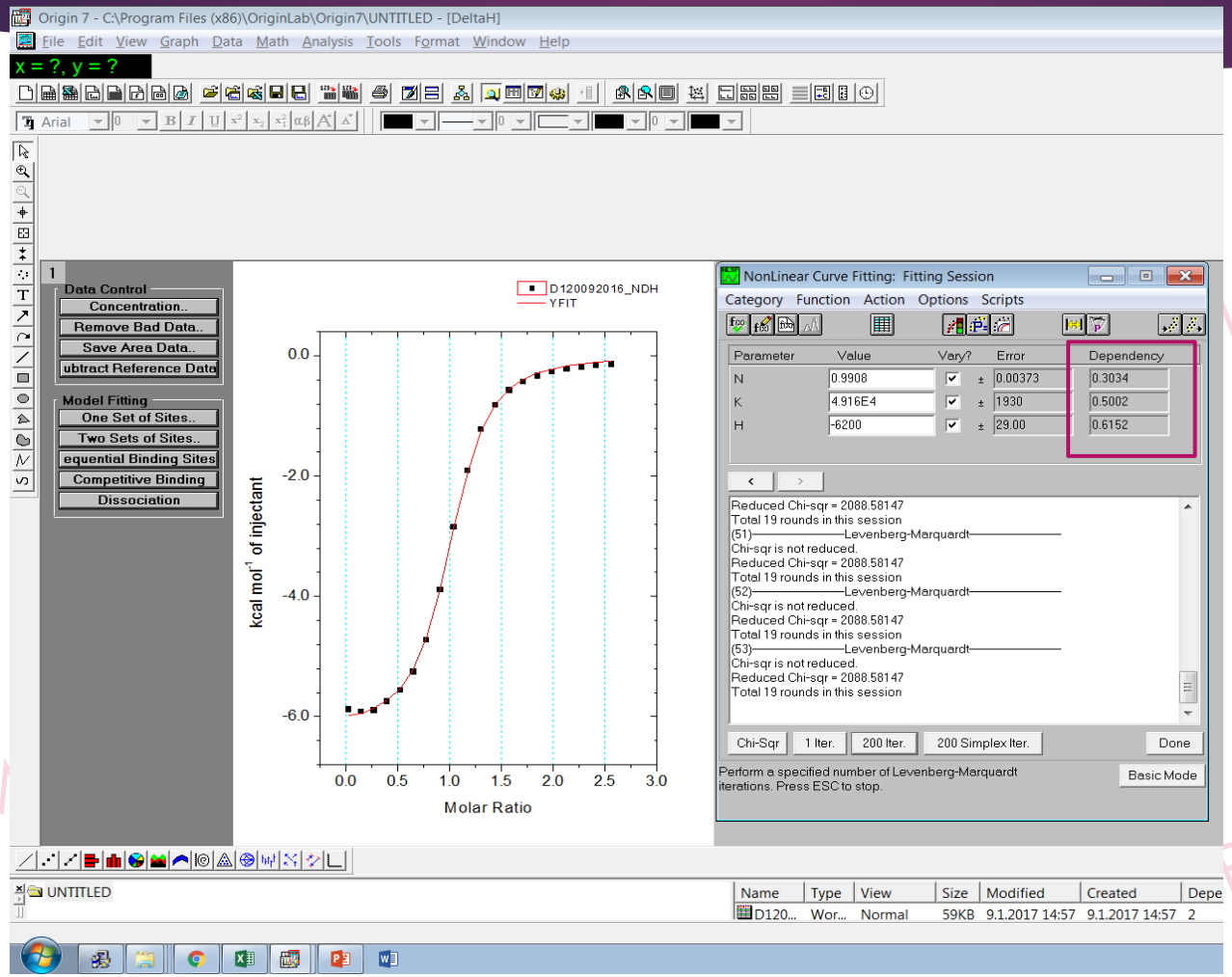
Delta – controls the way partial derivatives are calculated

Parameter constrains – allow to exclude unphysical values of parameters (USE WITH CAUTION)





Quality of fit: dependency of parameters



✓**Dependency value very close to 1 indicates strong cross-correlation and over-parameterization.**

Quality of the fit

- Chi-sq
- Parameter dependence
- Errors in the fitted parameters
- Agreement between repeated experiments
- Biochemical and experimental relevance in the parameters returned by the fit

Quality of the fit: fitted parameters
N, number of binding sites

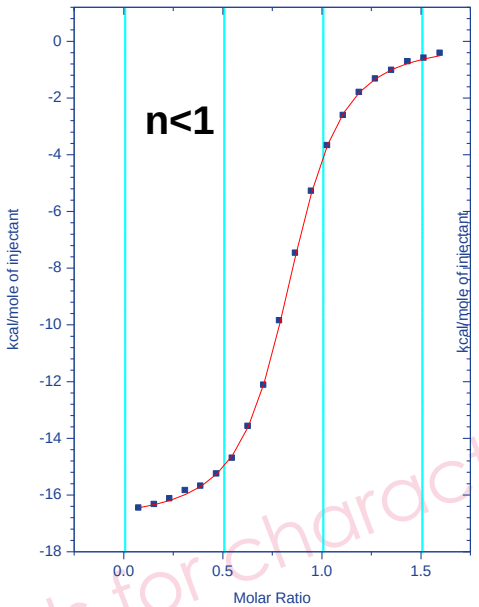
$$Q = \frac{nM_t \Delta HV_o}{2}\left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t}\right)^2 - \frac{4X_t}{nM_t}}\right]$$

- "N" is the average number of binding sites per mole of protein in solution, assuming:
  - that all binding sites are identical and independent
  - that you have pure protein (and ligand)
  - that you have given the correct protein and ligand concentrations
  - that all your protein is correctly folded and active

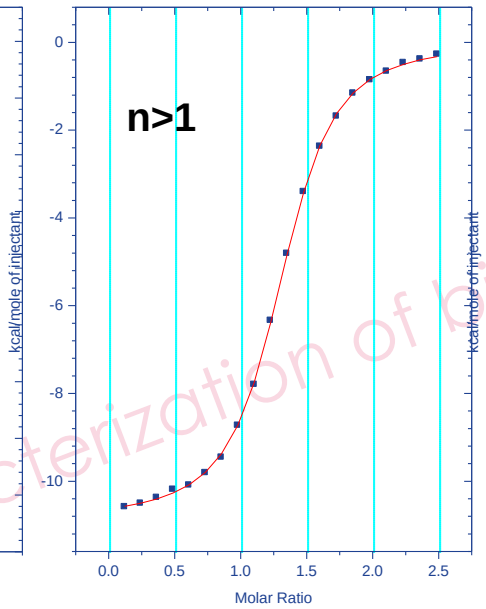
Goodness of the fit: fitted parameters *N, number of binding sites*

- If N≠ 1
- different number of binding sites
- inaccurate input values for protein and/or ligand concentration
- protein instability issues
- compound solubility issues
- binding does not fit simple independent model

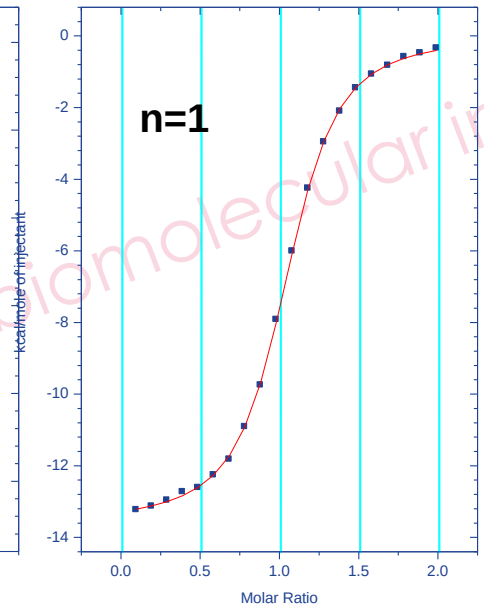
Stoichiometry: Incorrect [Ligand]



N 0.8218
K 6.899E4
ΔH -1.694E4

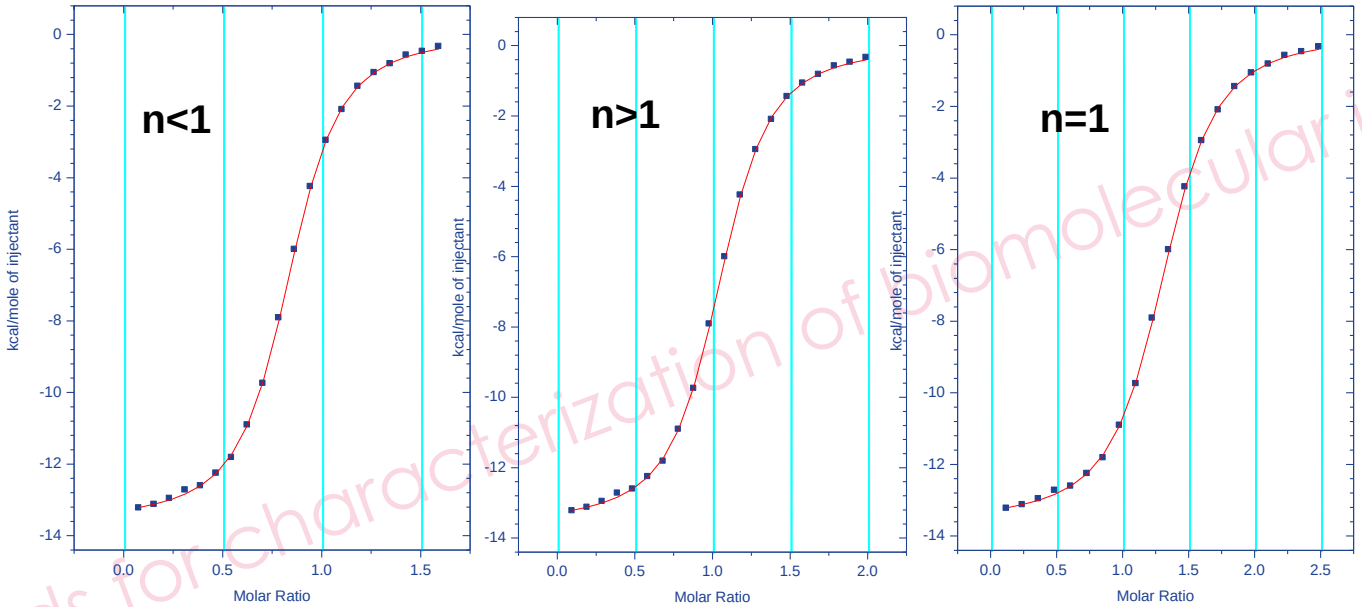


N 1.279
K 4.434E4
ΔH -1.089E4



N 1.023
K 5.543E4
ΔH -1.361E4

Stoichiometry: Incorrect [Protein]



N 0.8181
K 5.543E4
ΔH -1.361E4

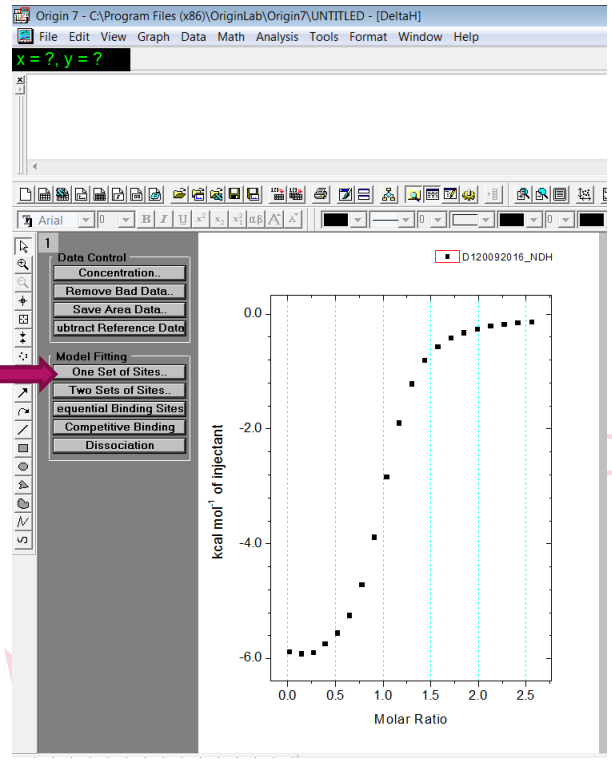
N 1.278
K 5.543E4
ΔH -1.361E4

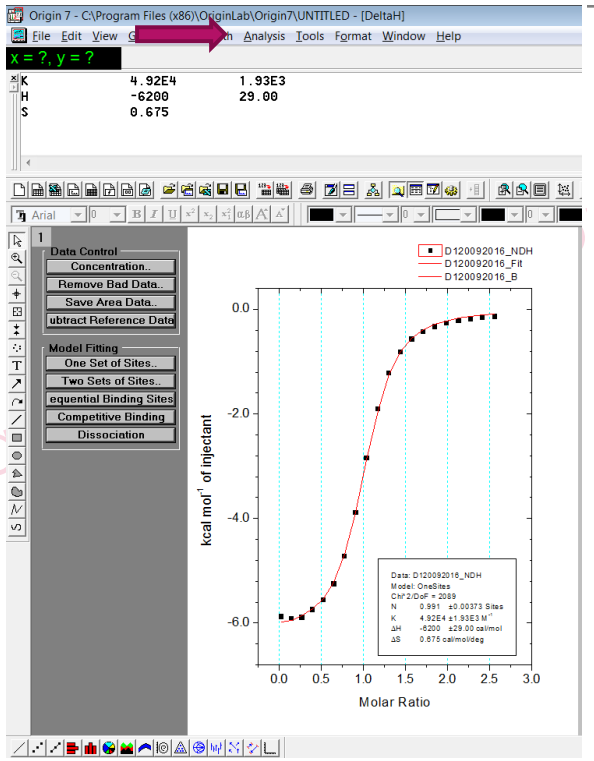
N 1.023
K 5.543E4
ΔH -1.361E4

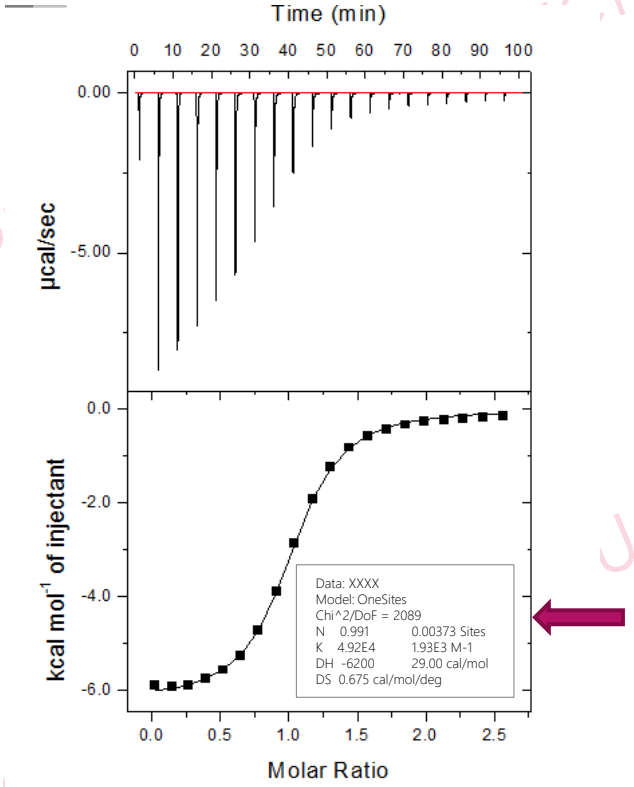
!!!

- **Error in syringe concentration results in error in DH, K and N**
- **Error in cell concentration results in error in N**
- Put the sample of which you have most control over in the syringe and evaluate accordingly

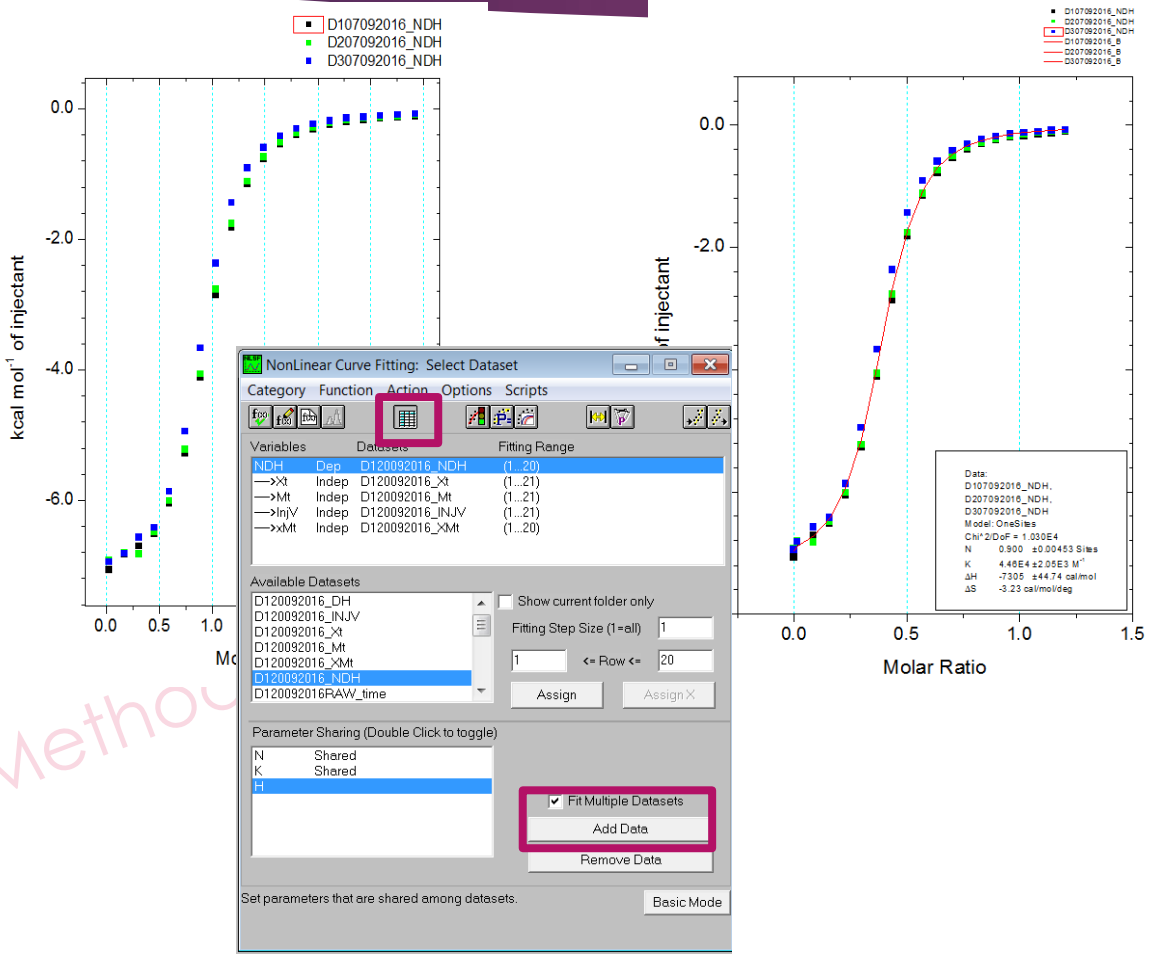
Reporting results: final figure and parameter box





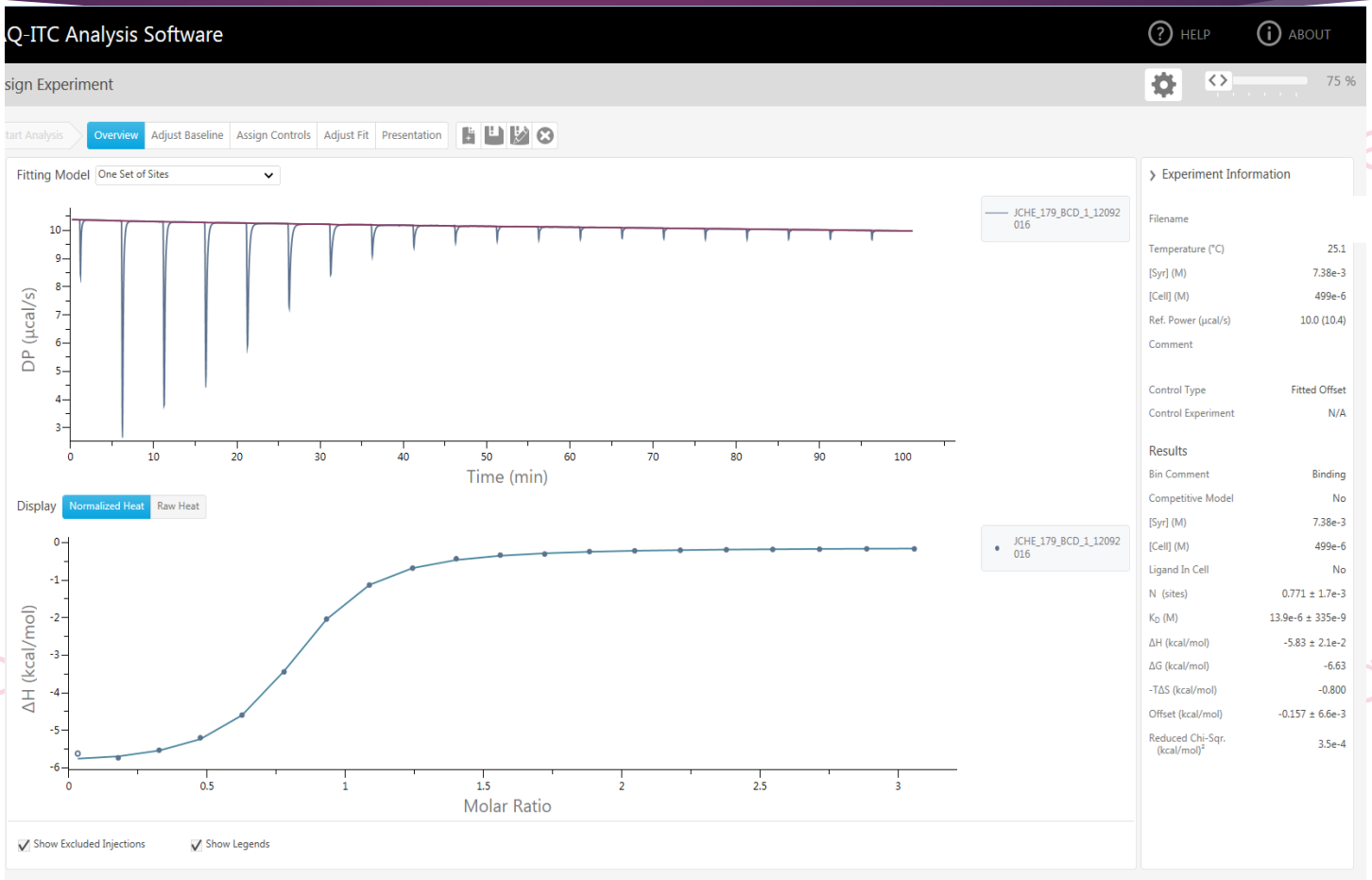


Global fit of multiple datasets collected at different experimental conditions

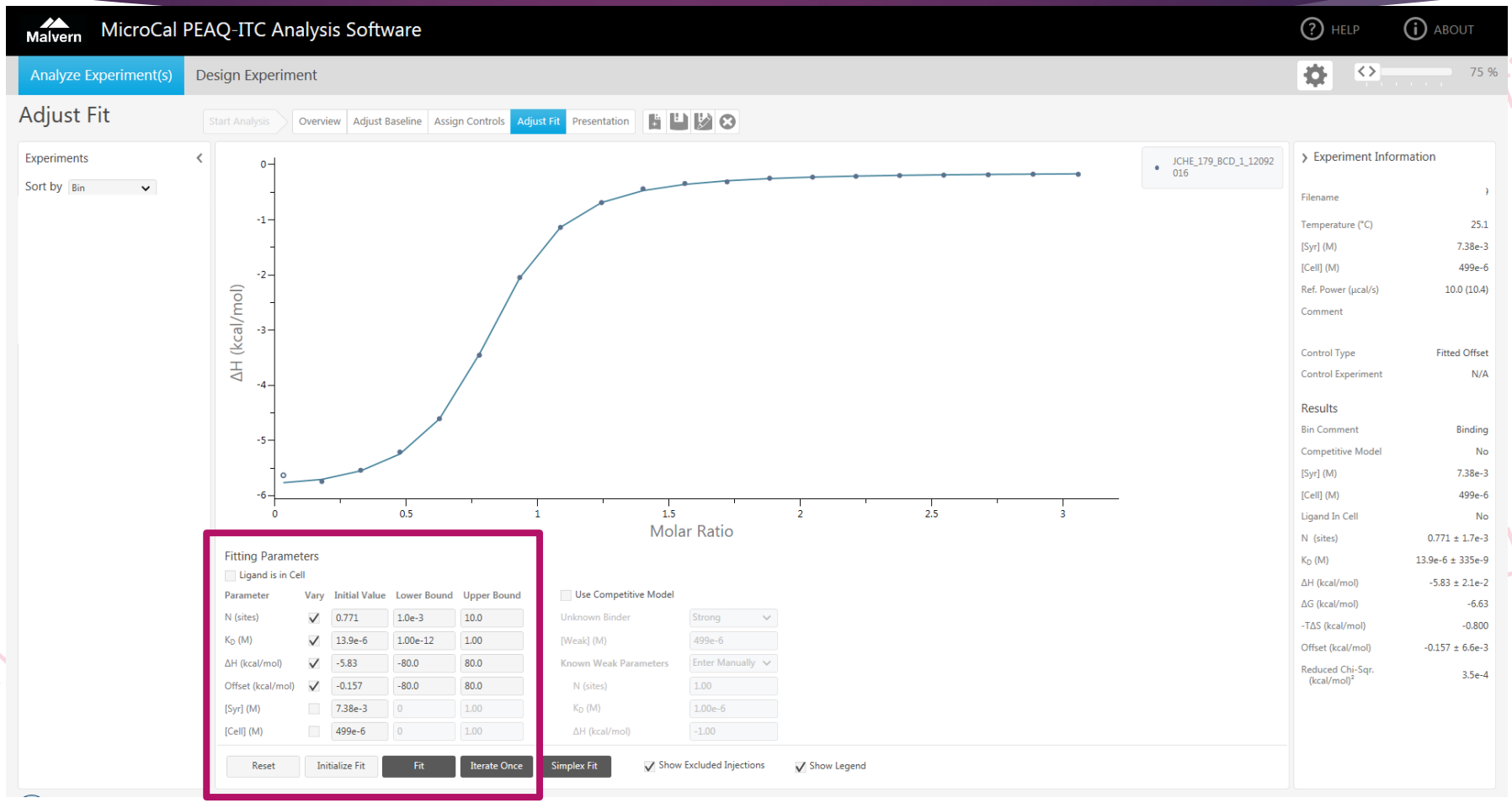


MicroCal PEAQ software-based ITC data analysis

MicroCal PEAQ-ITC software

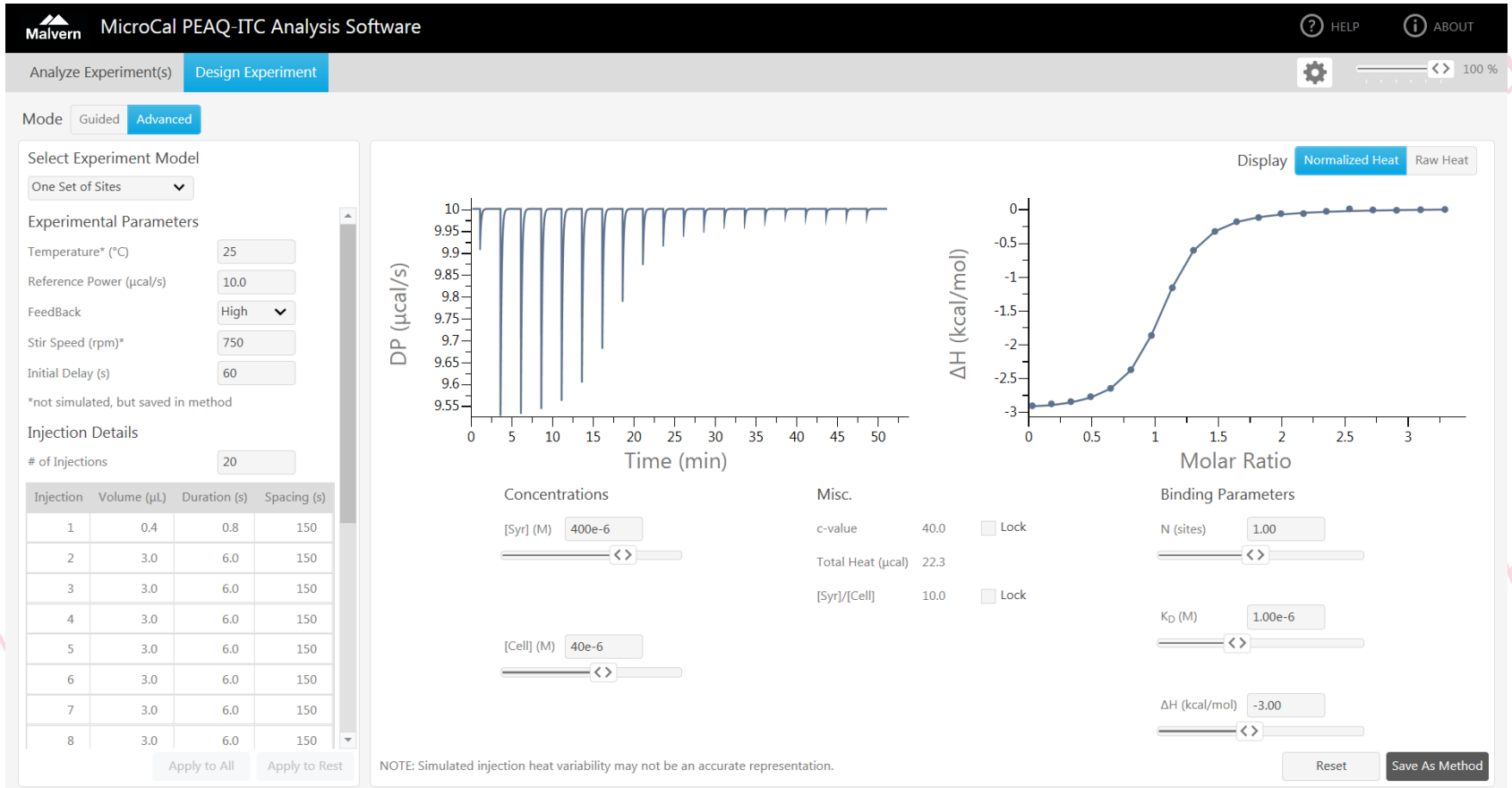


MicroCal PEAQ-ITC software



MicroCal PEAQ-ITC software

MicroCal PEAQ-ITC software – Design experiment



Thank you for you attention...☺

ITC and **DSC** techniques available in **CF BIC** (Core Facility of Biomolecular Interaction and Crystalization) **bic.ceitec.cz**
CF Head: Prof. Michaela Wimmerová
Contact: Monika Kubíčková, monika.kubickova@ceitec.cz C04/339

Data fit: non-linear least-squares minimisation

- Fitting procedure evaluates the deviation of the fitted function from the experimental data in terms of chi-squared.

- In Origin ITC data analysis module minimization is performed iteratively by Marquardt-Levenberg or simplex algorithms