Microcalorimetry

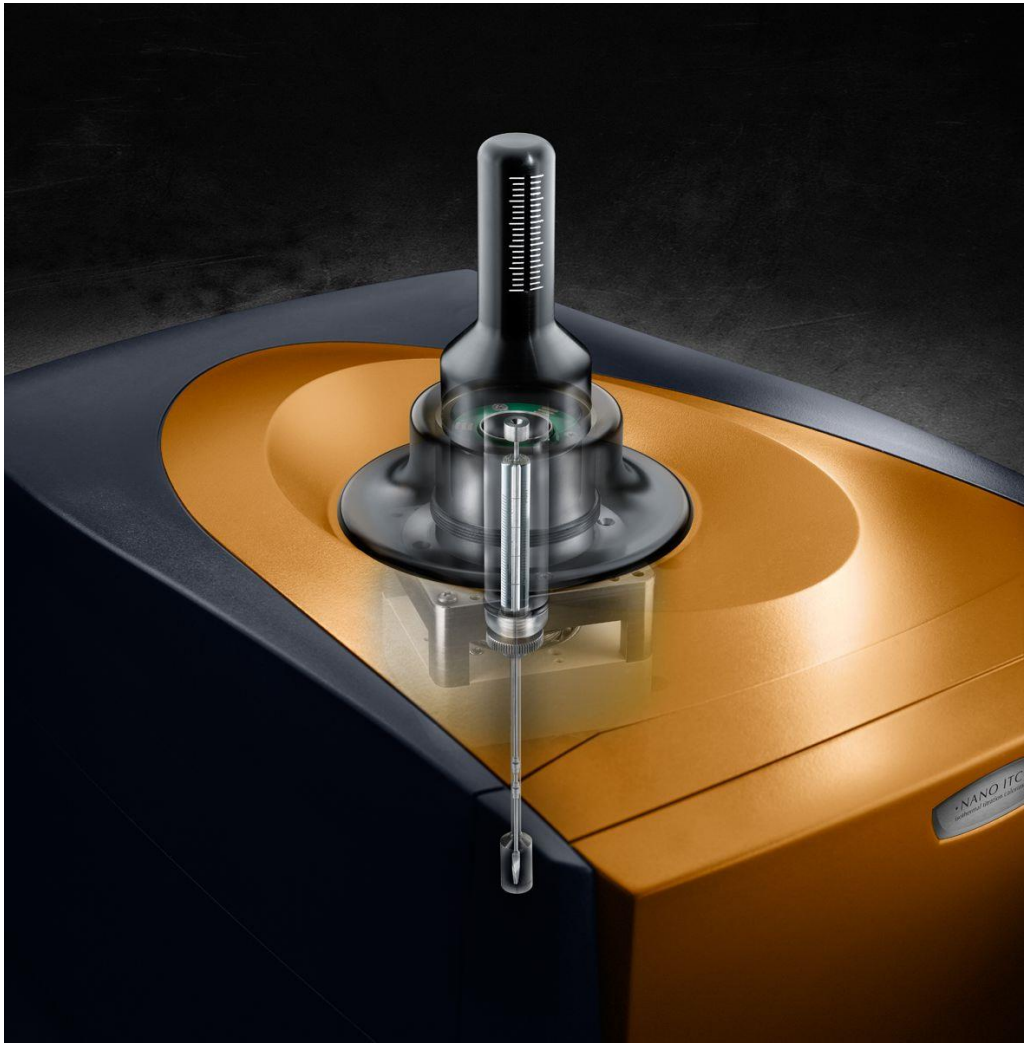
Introduction to characterizing biopolymer binding and kinetics reactions by ITC

Why use ITC?

- Completely general technique
  - Provides information on thermodynamics of the reaction
- Technique of choice for affinity measurements
  - Natural, unmodified ligands and substrates. No immobilization
- Equally useful for macromolecules and small molecules
- Compatible with essentially any buffer or additive
- Reactions conducted isothermally at any temperature between 2 to 80 ^{o}C

Nano ITC

A Choice of Sample Volume Cells with Nano ITC

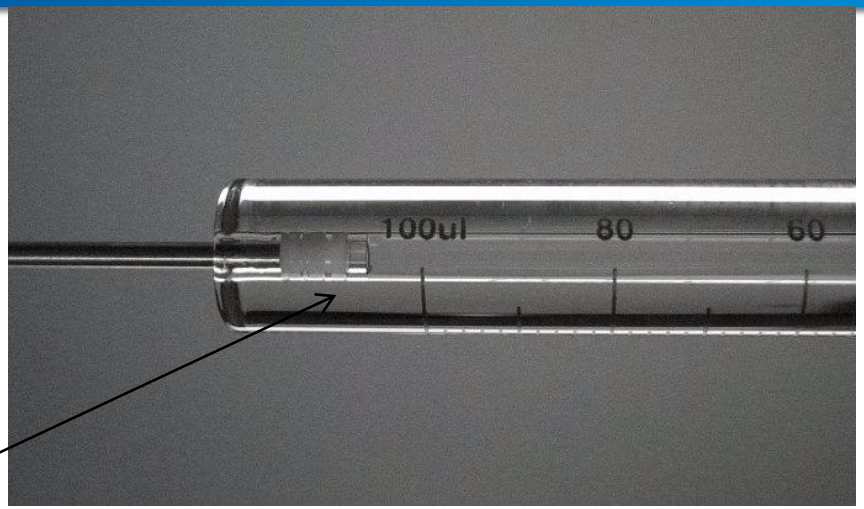


Nano ITC - Burette and Titration Syringe

Standard Volume (1 mL) – 100 μL and 250 μL syringe

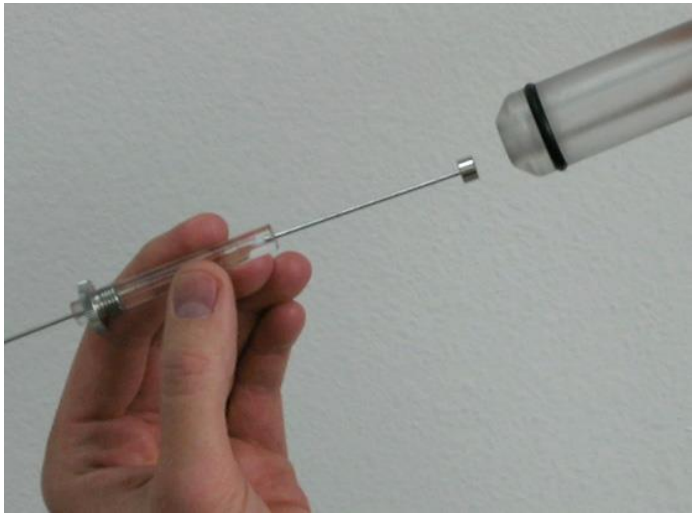
Low Volume (190 μL) – 50 μL syringe

Titration Syringe

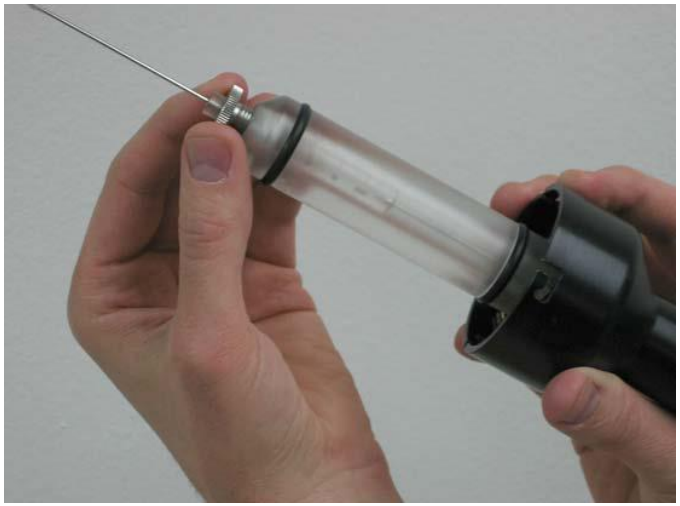


Air bubble

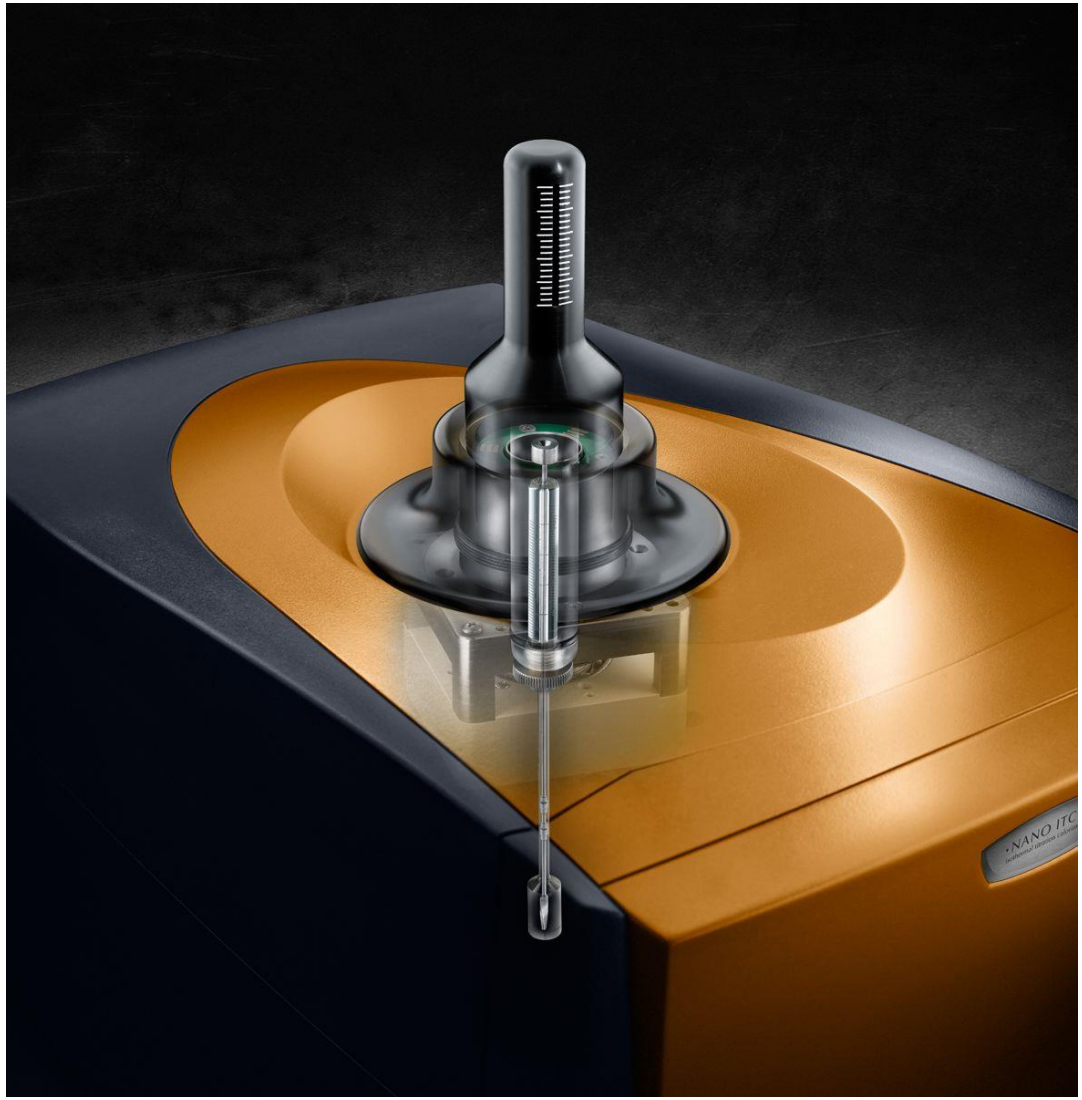
100 µL Syringe



Burette & Syringe Assembly



Nano ITC Ready for Titration



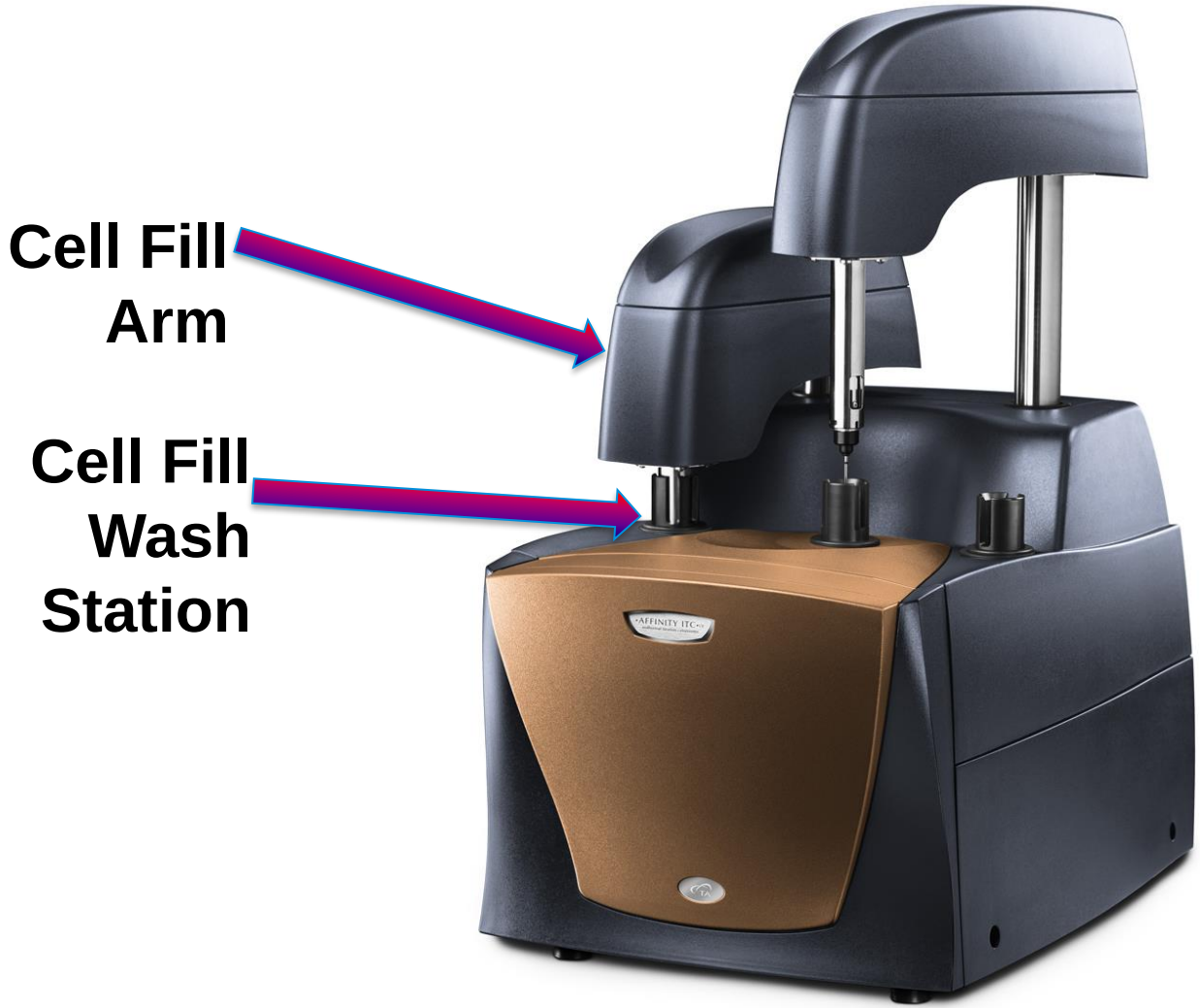
- Guide the buret with injection needle, needle first, into the top opening of the calorimeter
- Gently push the buret handle downward and rotate it slightly clockwise to secure the buret in place

New Affinity ITC Instruments

Affinity ITC

Affinity ITC Auto

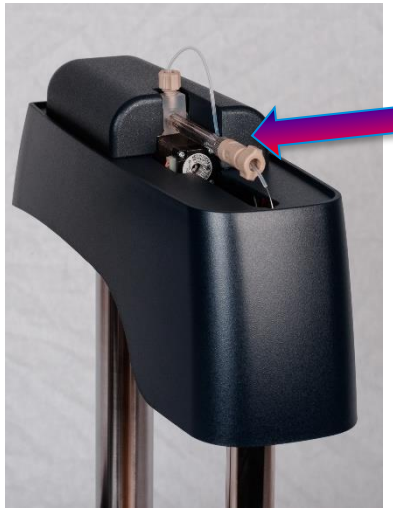
Affinity ITC Auto



Semi-Auto Affinity ITC

Injection Technology

- AccuShot Hardware:
  - Sample injection cannula separate from stirring mechanism
  - Small cannula (0.010” ID)
  - High precision stepper motor for injection syringe plunger ensures accurate volume delivery (Inj vol precision = 0.01 µL)
- AccuShot Control:
  - User Selectable injection volumes in ITCRun
  - Last minute editing of injection volumes and spacing ensures optimum use of reagents
  - Save and retrieve experiment templates



Injection Syringe: 250 µL syringe for SV and LV

Injection Cannula: Delivers sample at top of stirring paddle

FlexSpin™ Stirring Technology

Nano ITC - Specification Comparison

	SV Nano ITC (Gold or Hastelloy Reaction Vessel)	LV Nano ITC (Gold Reaction Vessel)
Min Detectable Heat (μJ)	0.1	0.05
Response time (s)	13 / 18	11
Recommended Stirring Speed (rpm)	350 / 250	350
Short Term Noise (μW)	0.0025	0.0014
Syringe Volume (μL)	250 or 100	50
Minimum Injection Volume (μL)	0.26 or 0.12	0.06
Temp. Stability (μºC at 25ºC)	50	50

Affinity ITC - Specification Comparison

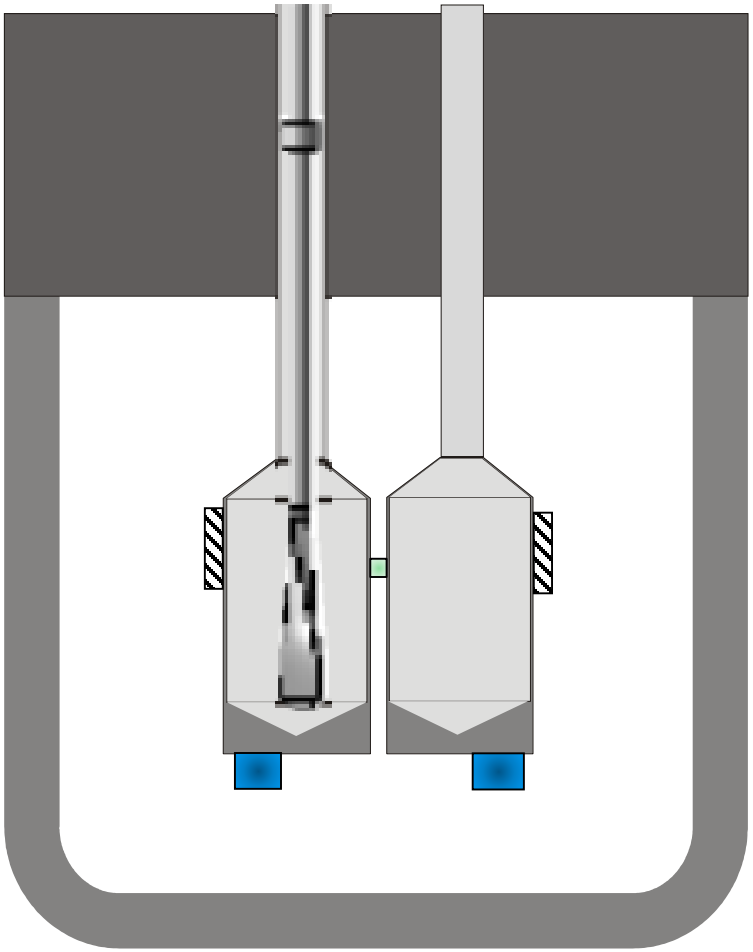
	SV Affinity ITC (Gold / Hastelloy Reaction Vessel)	LV Affinty ITC (Gold / Hastelloy Reaction Vessel)
Min Detectable Heat (μJ)	0.1	0.04 / 0.05
Response time (s)	13 / 18	3.3 / 11
Recommended Stirring Speed (rpm)	125	125
Short Term Noise (μW)	0.0025	0.0013 / 0.0014
Injection Syringe Volume (μL)	up to 250	Up to 250
Injection Volume Precision (μL)	0.01	0.01
Temp. Stability (μºC at 25ºC)	50	8 / 50

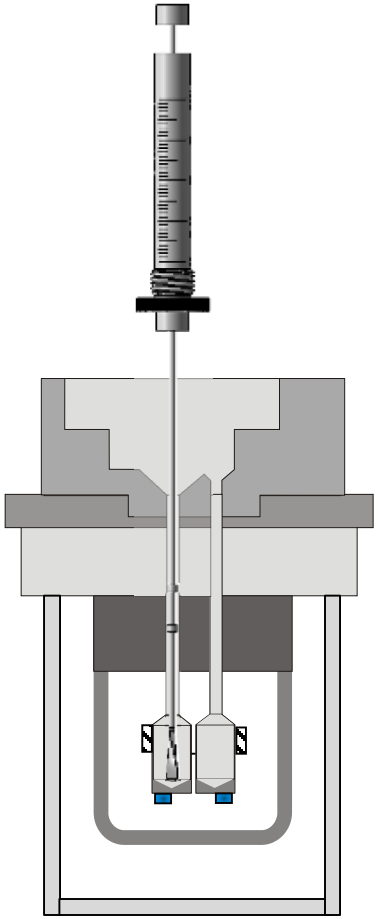
Nano and Affinity ITC – Same specs

	SV Affinity ITC (Gold / Hastelloy Reaction Vessel)	LV Affinty ITC (Gold / Hastelloy Reaction Vessel)
Active Cell Volume (mL)	1.0	0.190
Minimum Load Volume (mL)	1.2	0.25
Baseline Stability (μW/hr)	0.02	0.02
Cell Geometry	Fixed Cylindrical	Fixed Cylindrical
Temp. Control	Active heating and cooling	Active heating and cooling
Temp. Range (° C)	2 – 80	2 - 80

Sample and Reference Cell Configuration

ITC cells





Basics of ITC

1. Mix two solutions
2. Measure the Heat (q)
3. Fit data using an assumed model

Quantify:

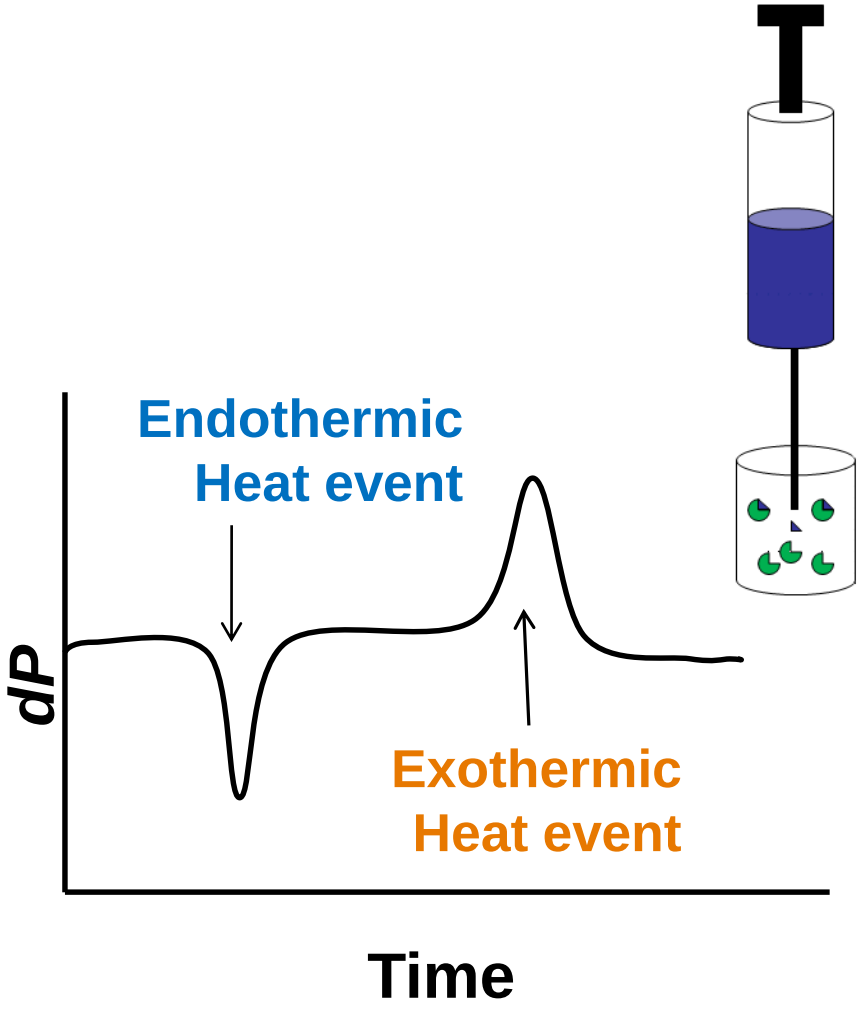
K_d, ΔG, ΔH, ΔS, stoichiometry, CMC
ΔC_p, $\Delta[H^+]$,
K_m, k_{cat}

4. Analyze data

Rationalize:

Structural changes

Lead optimization

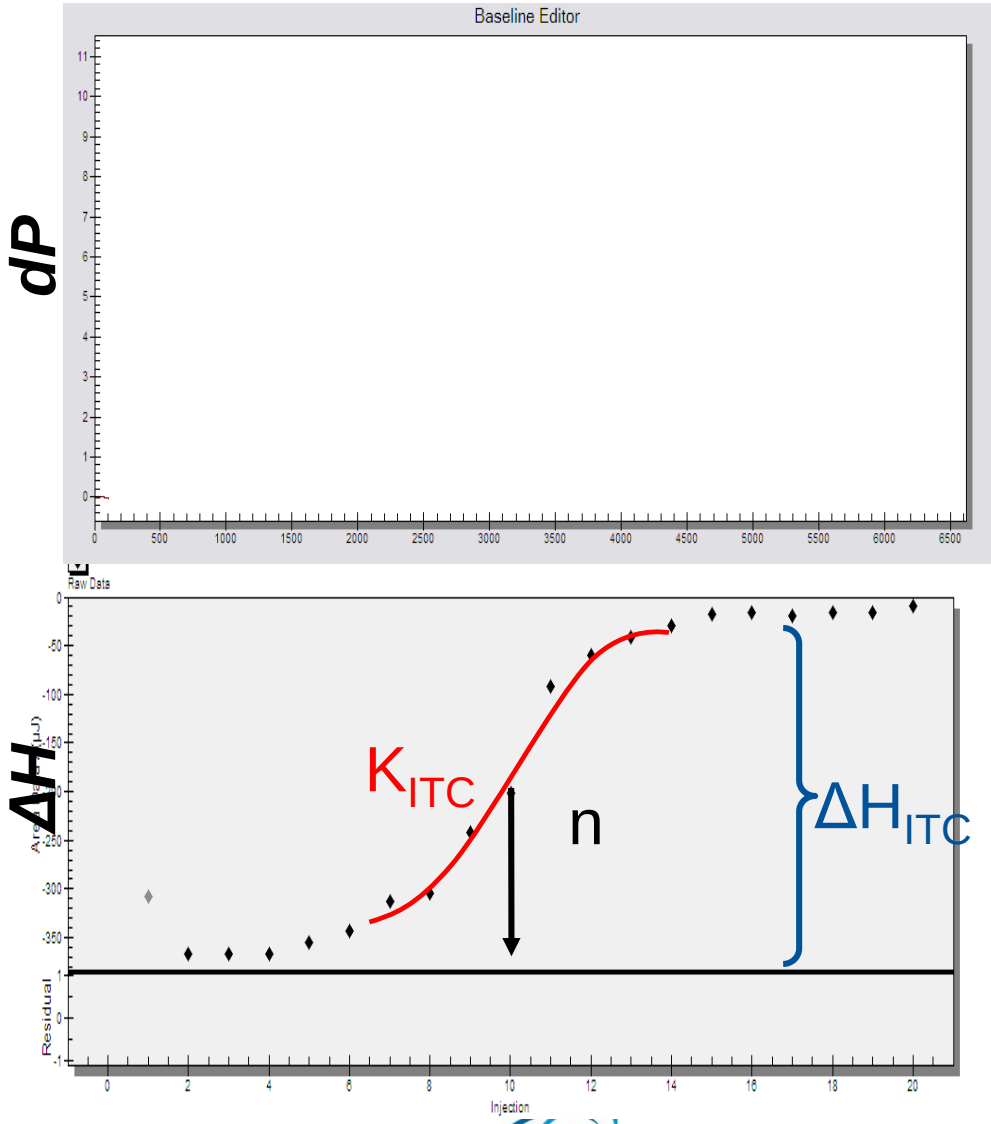


ITC Thermogram

- Fit of the data provides:
  - Enthalpy (ΔH_{ITC})
  - Stoichiometry (n)
  - Binding constant

    ($10^3 < K_{ITC} < 10^8$)

- Thermodynamic parameters:
  - $\Delta G = - RT \ln(K)$
  - $\Delta G = \Delta H - T \Delta S$
- Heat Capacity
  - $\Delta C_p = (\partial \Delta H / \partial T)_p$



Prior to Any Study: ITC Calibration

- "Calibration of nanowatt isothermal titration calorimeters with overflow reaction vessels"

- *Analytical Biochemistry* 417 (2011) 247-255

- Addresses:

1. Chemical calibration
   - HCl into Tris
2. Cell volume calibration
   - HCl into $KHCO_3$
3. Injection volume calibration
   - Mass Difference

WHY ITC?

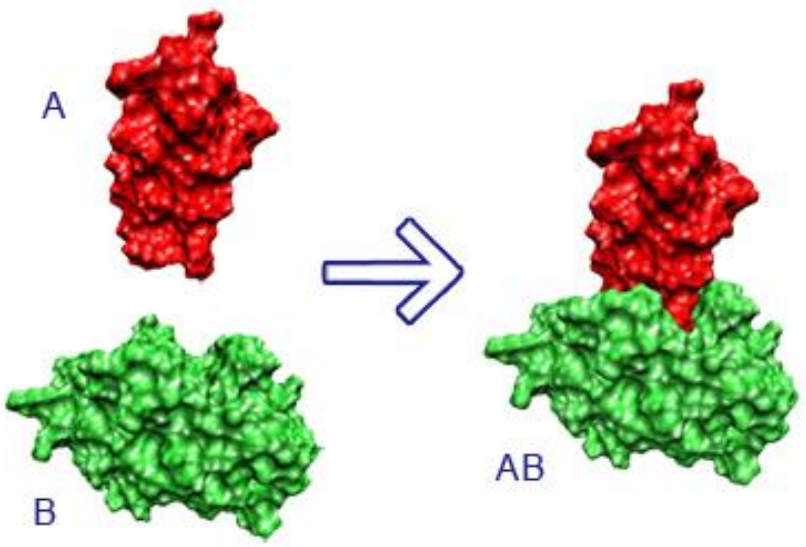
- Binding between macromolecules:
  - binding constant
  - stoichiometry
  - enthalpy
  - entropy
  - free energy
- Competition experiments
- Binding to complex macromolecular targets (e.g., higher order complexes/liposomes)
- Nanomaterial association
- Enzyme kinetics

Experimental approaches are applicable to all macromolecules, not just proteins

Binding interactions

- Measure the affinity of binding between two or more molecules (complex)
  - Protein-Protein, Protein-DNA, Protein-RNA, Protein-Lipid
    Protein-Carbohydrate, Protein-Metal ion
  - DNA-DNA, DNA-RNA, DNA-Metal ion
  - Protein-Nanomaterial, DNA-Nanomaterial, Antibody-Nanomaterial
  - Etc. – Any interacting molecules

Protein-Protein Interaction



- The affinity of an interaction is a basic chemical property of the interaction.
- Researchers identify the affinity of an interaction, so they can then try and block/strengthen the interaction to influence a therapeutic-cellular response.
  - Drug discovery,
- Enthalpy and stoichiometry provide additional information
  - Type of binding (hydophobic/H-bonding)
  - Ratio of binding

Application: Incremental titration & binding

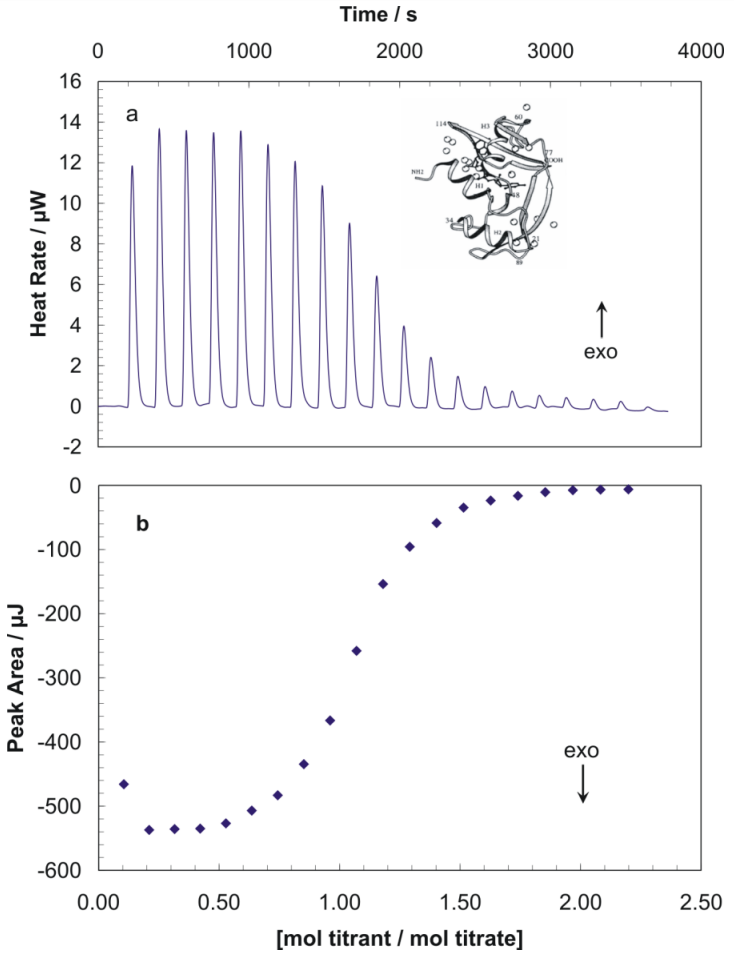
2’-CMP titrated into RNase A

Setup

- 1.6 mM 2’CMP (titrant)
- 80 µM Rnase A (titrand)
- 20 × 5 mL injections at 25 °C

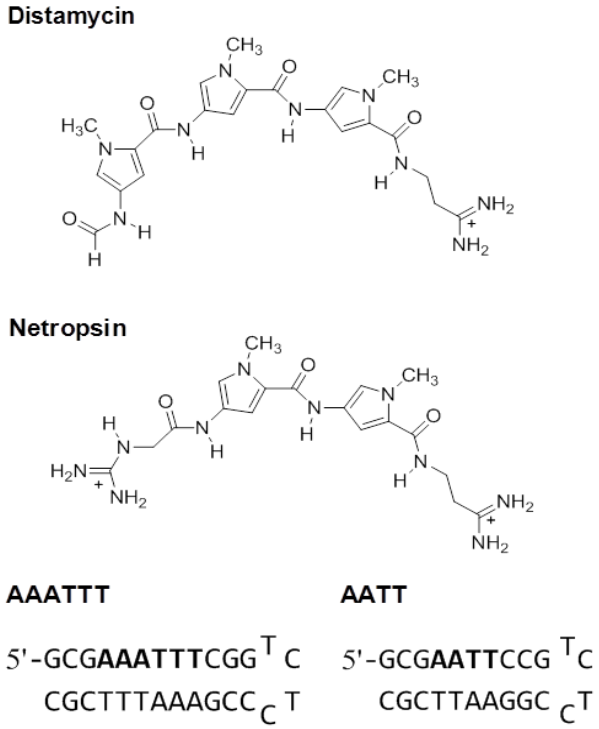
Results

- n = 1
- K_a = 1 x 10^6 M^{-1}
- Enthalpy of binding: -65 KJ/mol



Incremental & DNA-ligand binding

- Thermodynamic data defines the driving forces of a binding.
- This is a piece of information for fragment based drug design, biotherapeutic characterization and other process analysis steps where a subsequent toxicity or in vivo failure can be quite expensive.

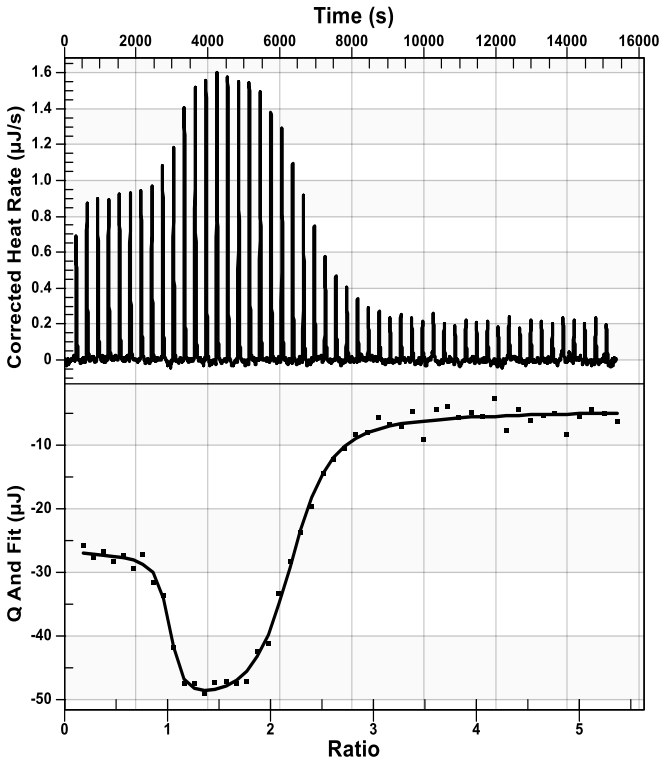


Anticancer agents. Alters DNA conformation

Application Note: Shuo Wang, Manoj M. Munde, PhD., W. David Wilson, PhD.,Georgia State University.

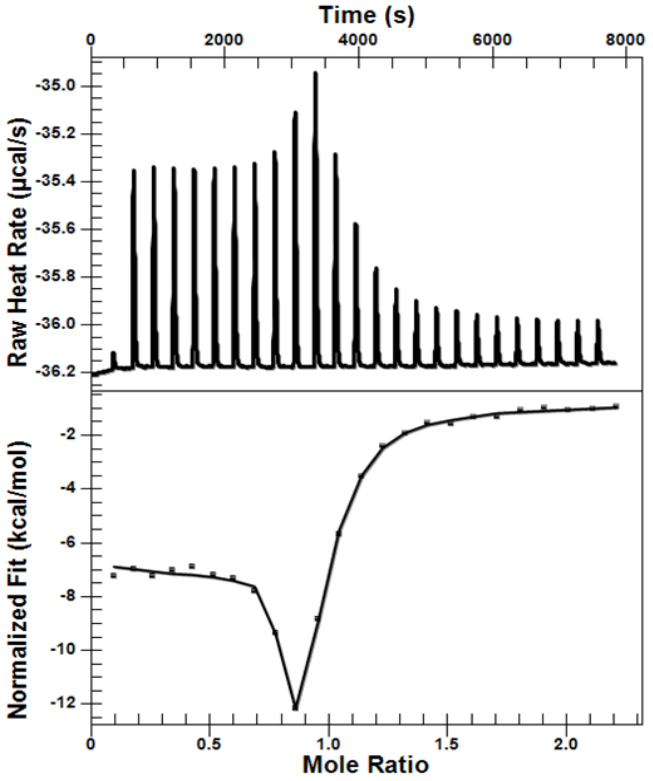
DNA Binding Studies

Distamycin → DNA



- SPR data indicated 1 site.
- 2D NMR & ITC data agree, 2 sites
- Negative cooperativity

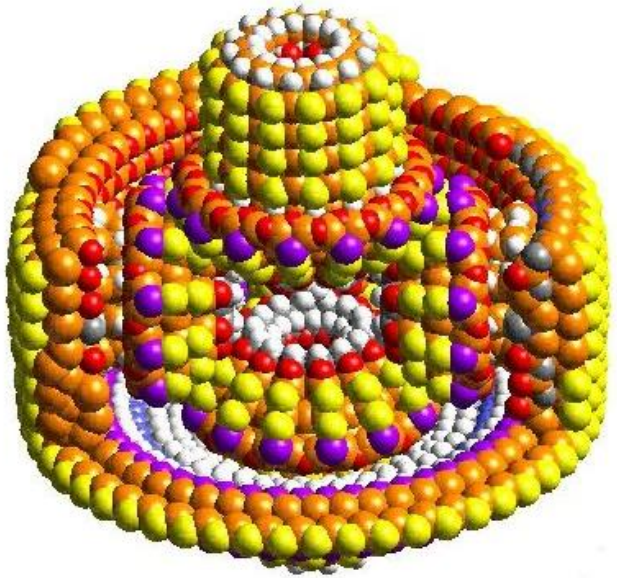
Netropsin → DNA

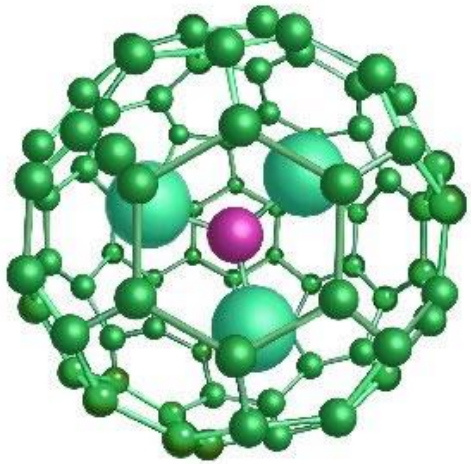


- Biphasic nature
  - Explanation: netropsin binds to the AATT site with and without a strongly bound water molecule.
  - 1:1 with different thermodynamics.

Application: Material Characterization

- **Micelle formation (CMC)**
- **Material Aggregation (CAC)**
- **Material Association/Association**
  - **Nanomachines**
- **Vesicle formation**



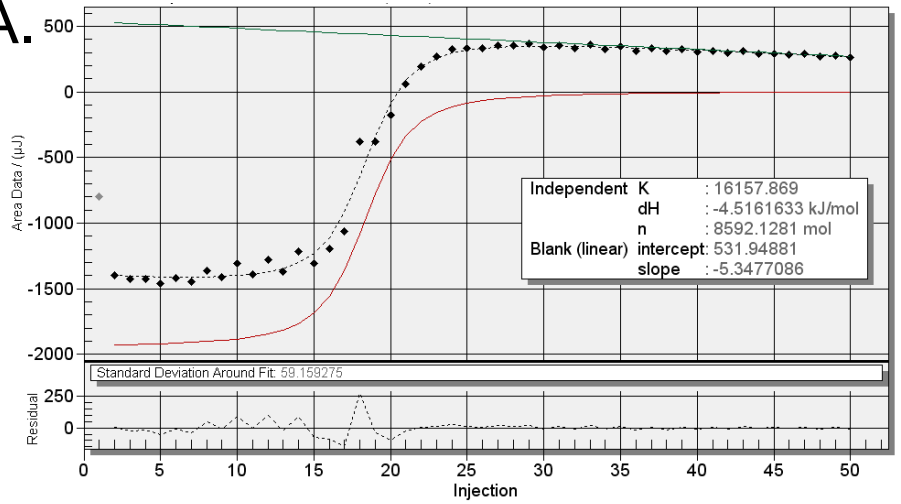


Nanomaterials are often coordinated with biological material and therapeutics as a novel approach to treat disease.

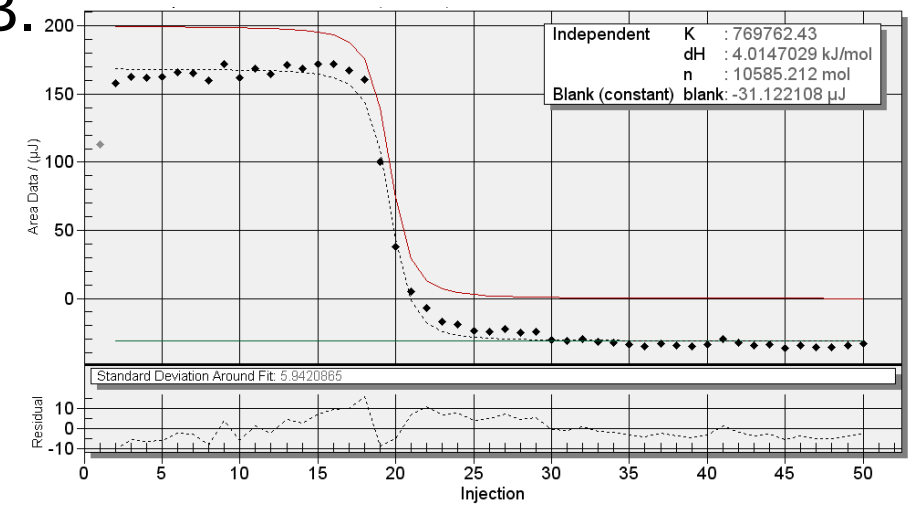
Determining the Critical Micelle Concentration

- Critical micelle concentration (CMC) is the concentration at which detergents aggregate to form micelles.
- Titrate concentrated detergent suspension (micelles) into buffer/water (**A**) SDS (**B**) CDAP.
- Initially micelles dissociate in sample cell. At CMC, detergent in the sample cell aggregates. Midpoint of the inflection is the CMC (n).

A.

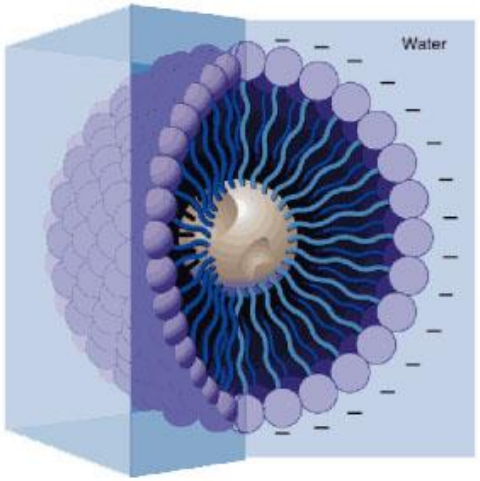


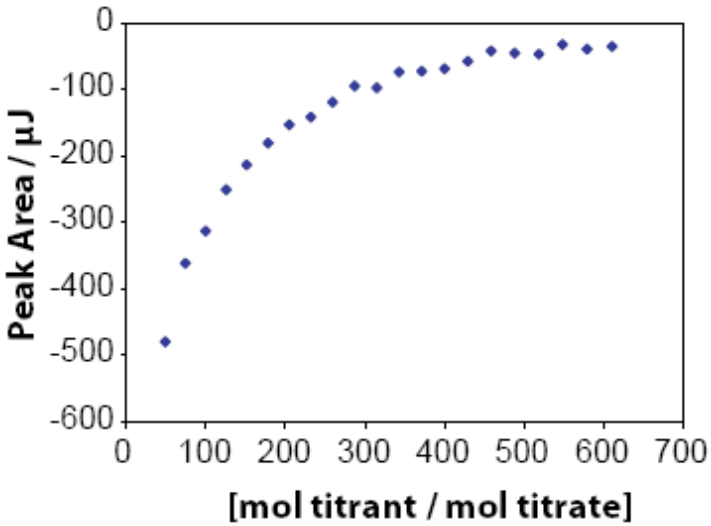
B.



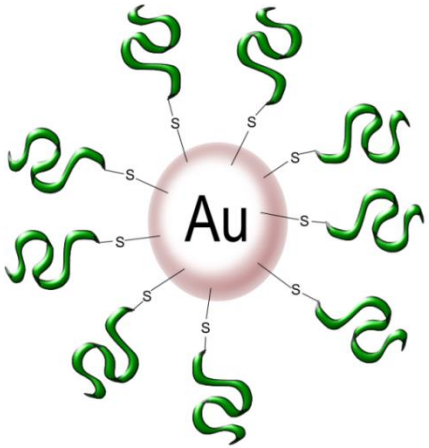
Application #3: Interactions with Vesicles or Micelles

- Macromolecule binding to vesicles/micelles often used as models for drug binding
- Binding (actually partitioning) is usually non-specific, so no fixed stoichiometry, but K_a and enthalpy can be determined
- Cyclosporin A titrated into DPPC vesicles
- K_a = 390 M^{-1}, ΔH = -61 kJ/mol DPPC
- Weak 'binding' consistent with partitioning

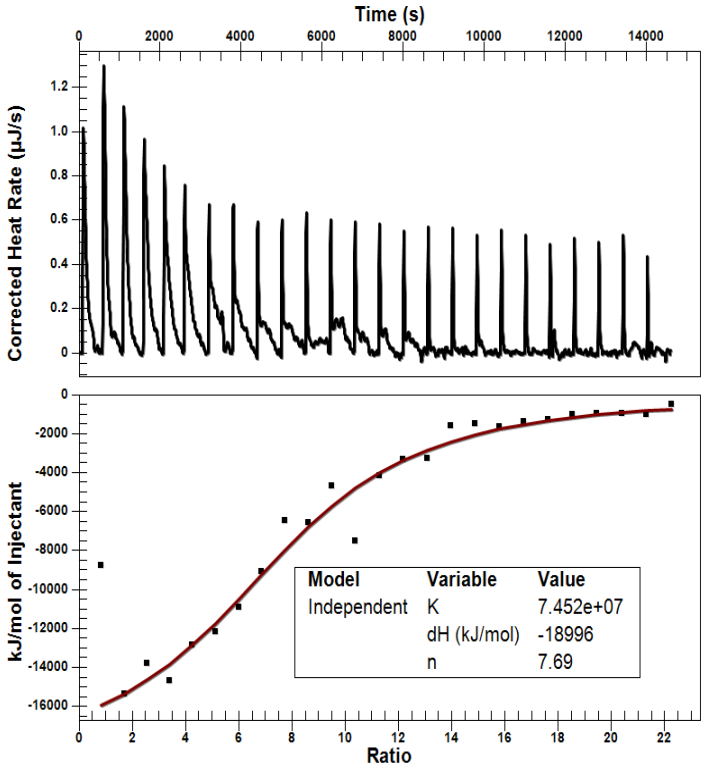




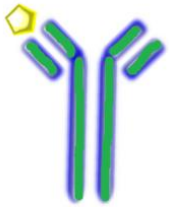
Application 4: Functionalizing Nanoparticles or other Macromolecular Assemblies with Antibodies



Titration of Antibody (µM) into Gold Nanoparticles (nM)



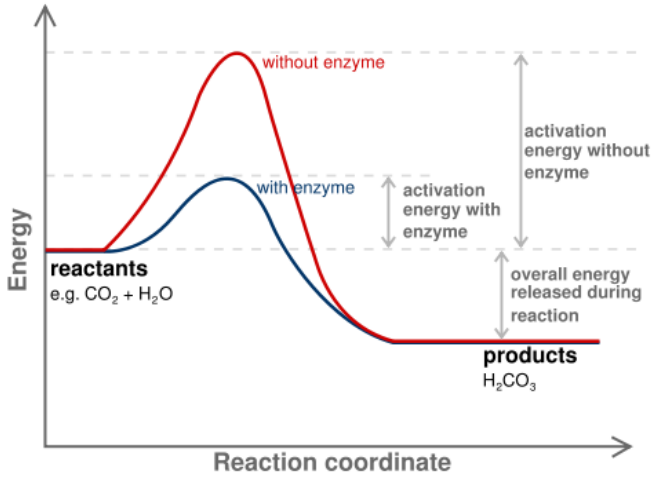
Next step after functionalizing the np with an antibody is binding of the antigen.



- Minimal detectable heat for the LV = 0.05 µJ and SV is 0.1 µJ
- LV ITC is 2x more sensitive than the SV, but the SV can hold 5x more moles (170 µL v 950 µL active volume).

Enzymes

- Macromolecules (proteins and RNA) that catalyze (*i.e.*, increase the rates of) chemical reaction
  - Kinase
  - Phosphatase
  - Oxidoreductase/Dehydrogesase
  - Ligase
  - Isomerase
  - Helicase



$$E + S \rightleftharpoons ES \rightleftharpoons E+P$$

- In enzymatic reactions, the molecules at the beginning of the process are called substrates, and the enzyme converts them into different molecules, called the products.

Enzyme Kinetics

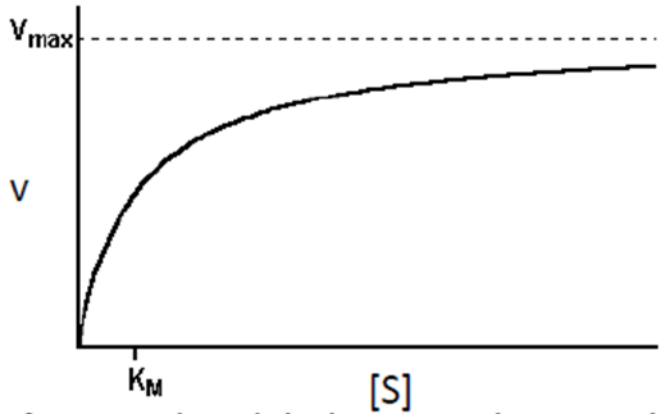
The rate or velocity (v) of the reaction is given by the Michaelis-Menten relationship:

$v = d[P]/dt = (v_{max}[S])/(K_M + [S])$

v_{max} = maximum velocity at saturating subtrate concentration [S]

K_M = Michaelis=Menten constant or the value of [S] at which v = ($V_{max}/2$)

[P] = concentration of the product released



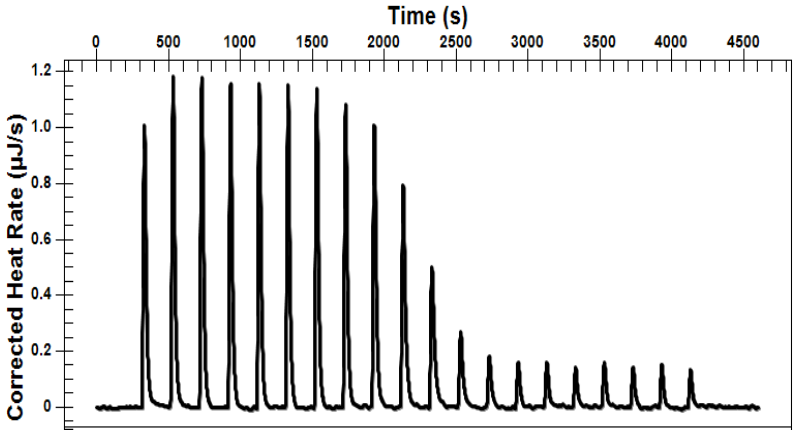
Enzyme turnover number (k_{cat}):

$k_{cat} = v_{max}[E]$

[E] = total enzyme concentrations

Application #5: Kinetic Studies Using ITC

The signal measured is thermal power
Power = dQ/dt, where Q = heat, t = time



Q (kJ) = nΔH
n= number of moles of product released
Δ H = enthalpy change associated with the rxn
n = V[P]
[P] = concentration of product released
V = reaction/cell volume

Therefore, Q = V ΔH[P] V and Δ H are constants – makes for easier math!

Differentiate with respect to time:

$$\frac{dQ}{dt} = V * \Delta H * \frac{d[P]}{dt}$$

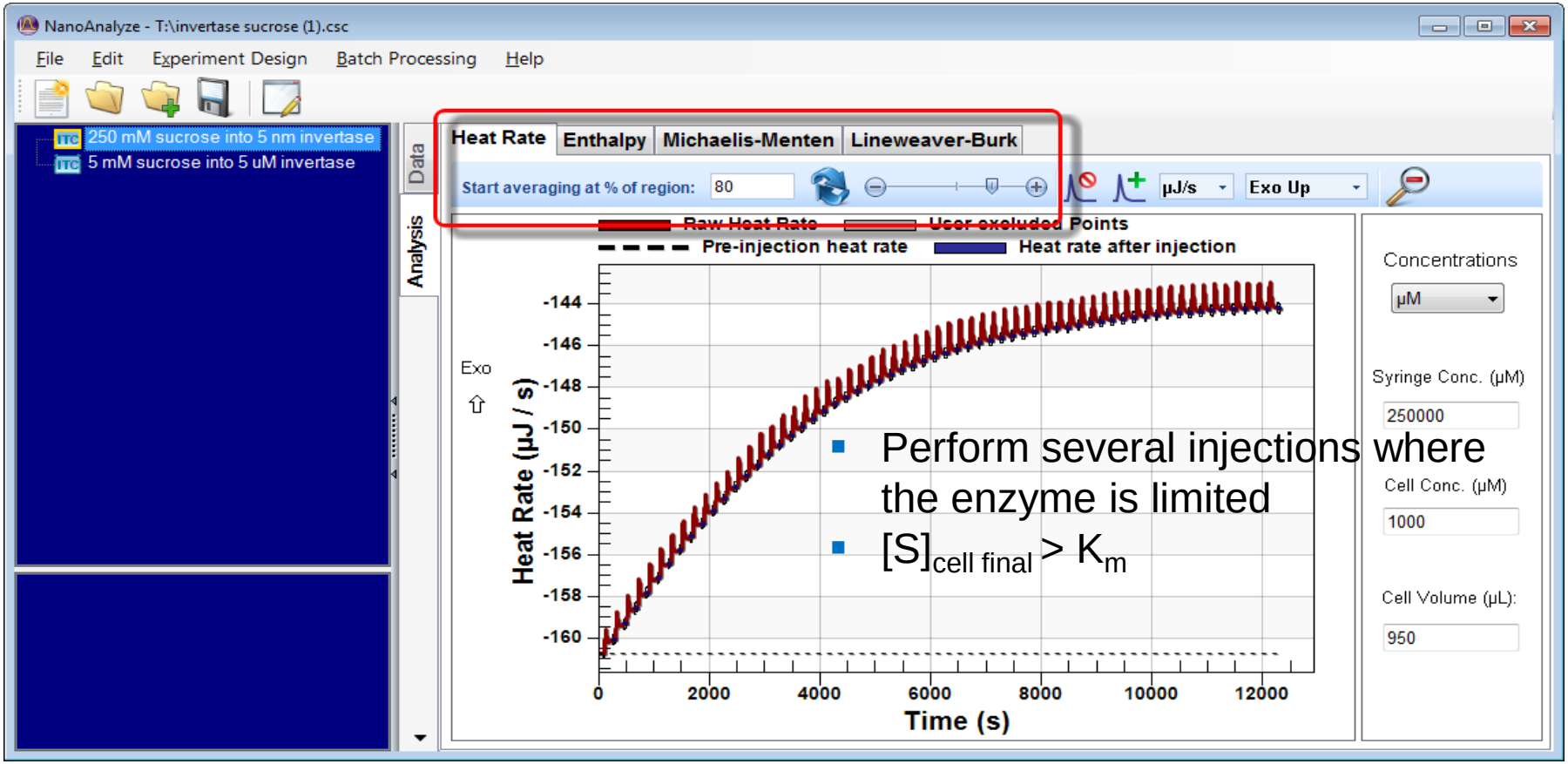
$$\frac{d[P]}{dt} = \frac{1}{V} * \frac{1}{\Delta H} * \frac{dQ}{dt}$$

$$\text{Rate(v)} = \frac{1}{V} * \frac{1}{\Delta H} * \frac{dQ}{dt}$$

The rate can be measured if [S] is known and [E] is in excess.
K_M, v_{max}, and k_{cat} can be subsequently determined from a plot of v vs [S]

MIM Enzyme Kinetics

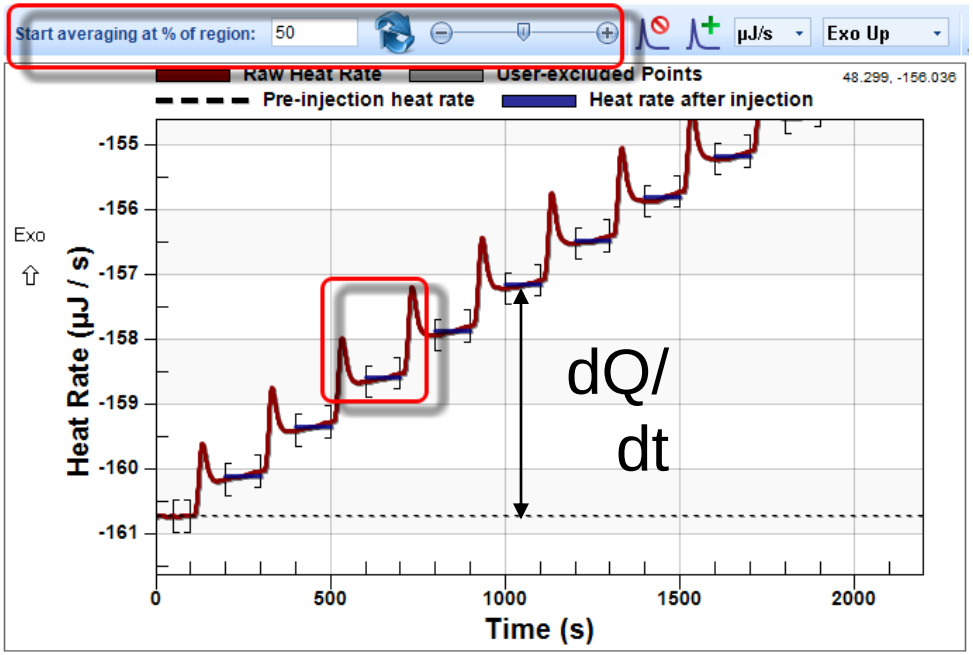
250 mM Sucrose → 5 nM enzyme



- Perform several injections where the enzyme is limited
- $[S]_{cell\ final} > K_m$

- In a successful titration final baseline plateaus.
- The plateau is related to the maximum turnover, or v_{max}.

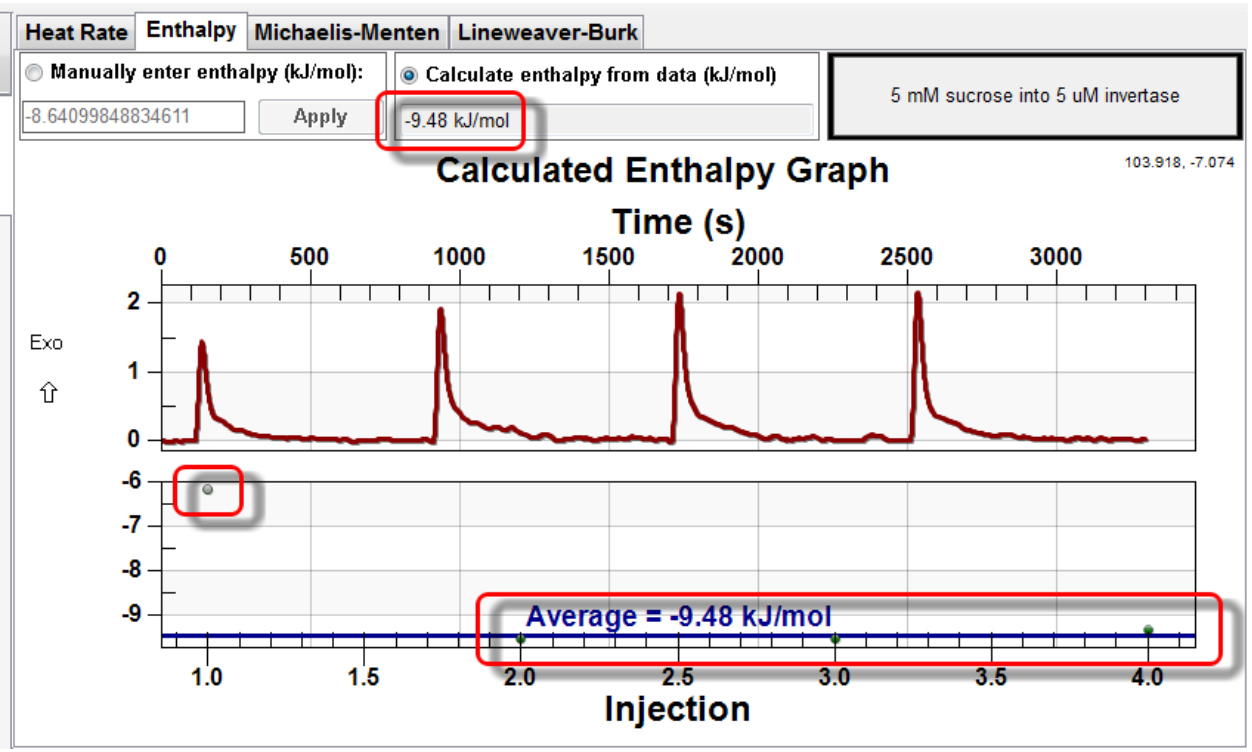
Determine Turnover (cont. 1)



1. Determine the differential power prior to the first injection.

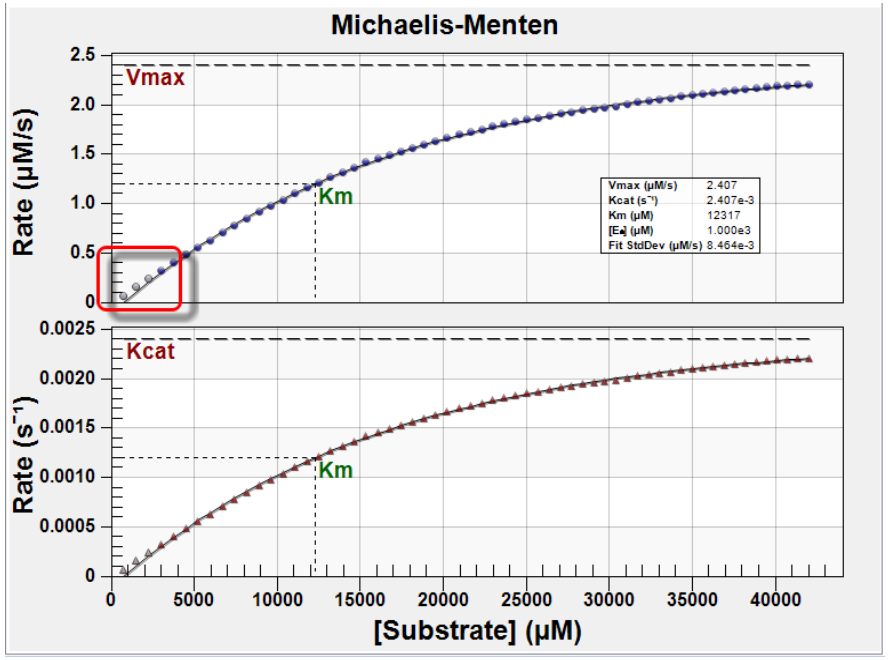
2. Determine baseline/differential power after the injection. The injection is NOT the event, the shift in the baseline IS the event. The baseline shifts because there is a continuous event (Substrate→Product) that is occurring that elevates the baseline.

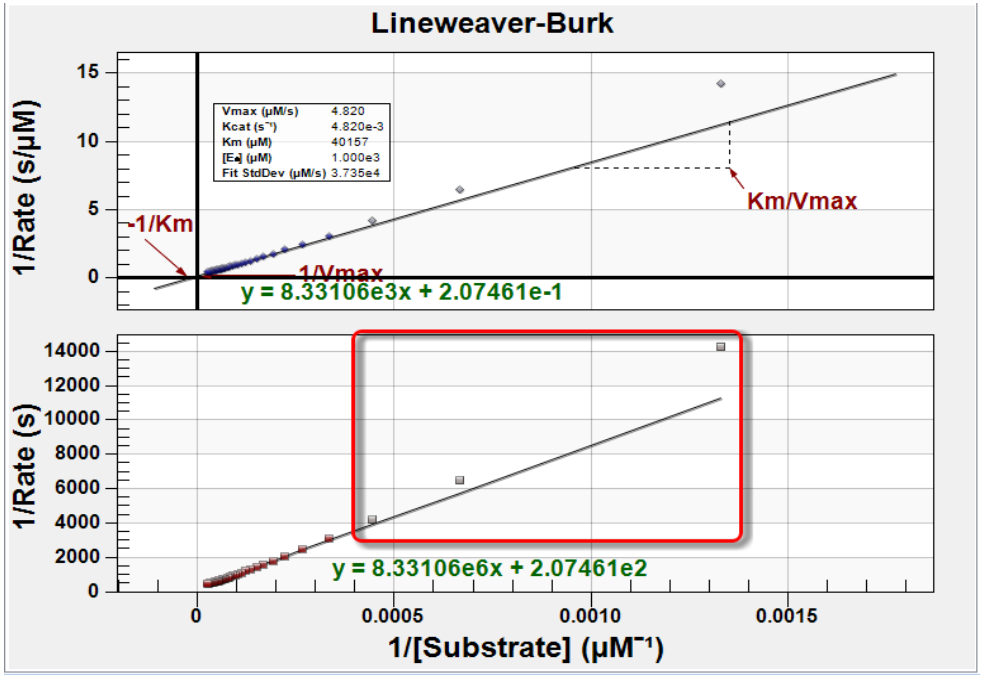
MIM Titration B: Determine Enthalpy



- Perform several injections where the enzyme will not be limited and all substrate is converted into product.
- For this case: 25 mM Sucrose → 7.4 µM enzyme
- All of the sucrose is turned into product and this titration measures the ΔH for this process.
- Note: plot normalized heat to get the kJ/mol value that is needed later.

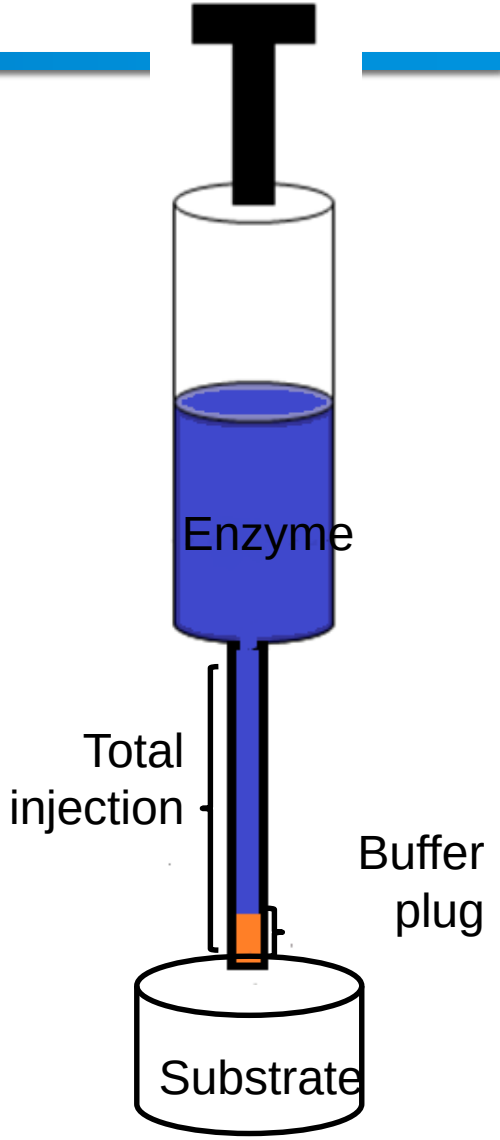
M-M or L-B Plots



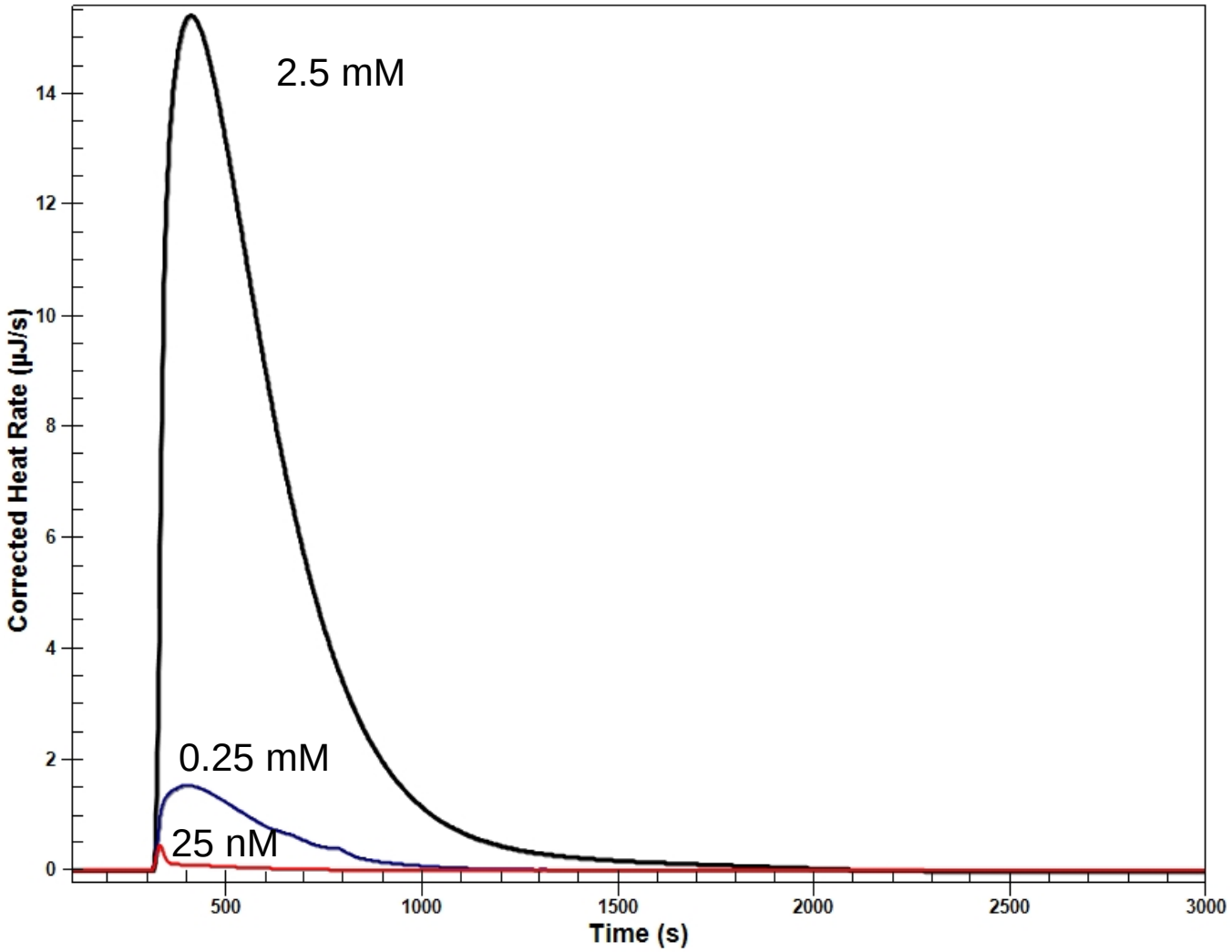


Single Injection Method (SIM) Kinetics

- Usually a moderate concentration of the substrate is used (mM or µM) and a relatively high concentration in of enzyme is in the syringe (µM or nM).
- To avoid starting the reaction early, the last few µL of the injection syringe are filled with buffer. The single injection is set-up to deliver the buffer plug and some enzyme
  - ie) 3 µL plug + 5 µL enzyme = 1 x 8 µL injection
- The heat flow (dQ/dt) is directly proportional to the rate with which the reaction is formed.
  - Most experiments will be completed in 1+ hour(s).
- Instrument response time consideration: design experiments where the ratio of enzyme to substrate in the calorimetric cell resulted in a reaction completion times at least one order of magnitude than the instrumental response time.
  - This typically means use more substrate



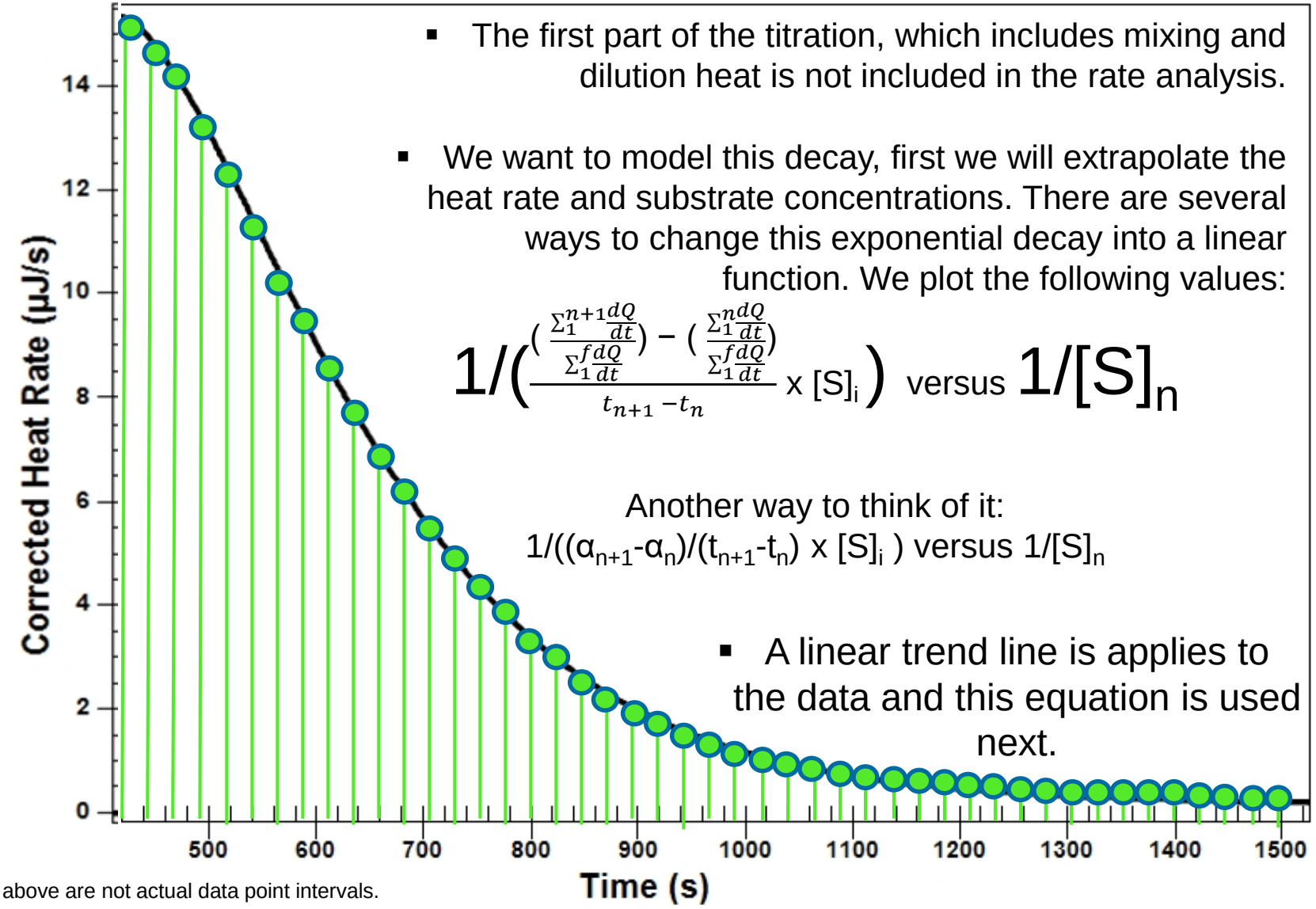
Invertase, Titrated into Varying Sucrose



5µL of 37 µM Invertase, titrated into varying concentrations of Sucrose.

100 mM Glycine Buffer pH 5.65

SIM Kinetics



- The first part of the titration, which includes mixing and dilution heat is not included in the rate analysis.
- We want to model this decay, first we will extrapolate the heat rate and substrate concentrations. There are several ways to change this exponential decay into a linear function. We plot the following values:

$$1/\left(\frac{\left(\frac{\sum_1^{n+1}\frac{dQ}{dt}}{\sum_1^{f}\frac{dQ}{dt}}\right)-\left(\frac{\sum_1^{n}\frac{dQ}{dt}}{\sum_1^{f}\frac{dQ}{dt}}\right)}{t_{n+1}-t_n}\times [S]_i\right) \text{ versus } 1/[S]_n$$

Another way to think of it:

$1/((\alpha_{n+1}-\alpha_n)/(t_{n+1}-t_n) \times [S]_i)$ versus $1/[S]_n$

- A linear trend line is applies to the data and this equation is used next.

Points above are not actual data point intervals.

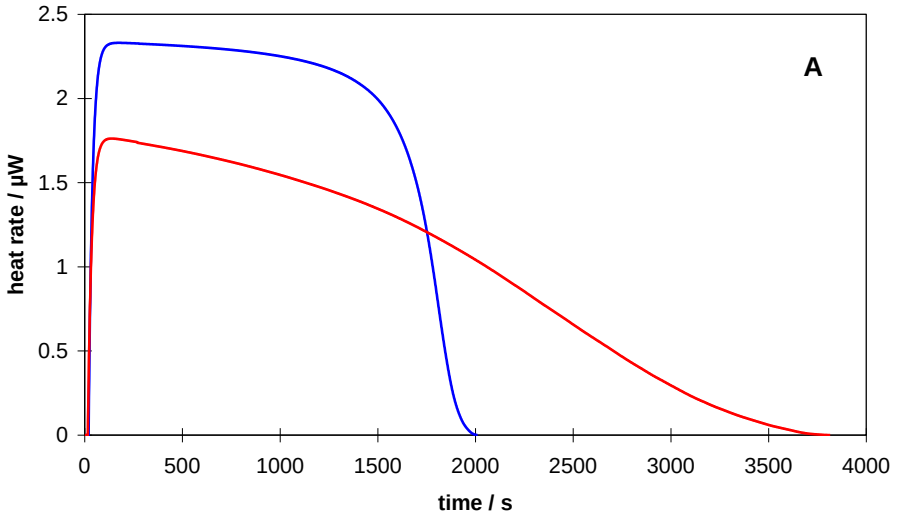
Application #5: Enzyme Kinetics and Enzyme Inhibition (Single Injection)

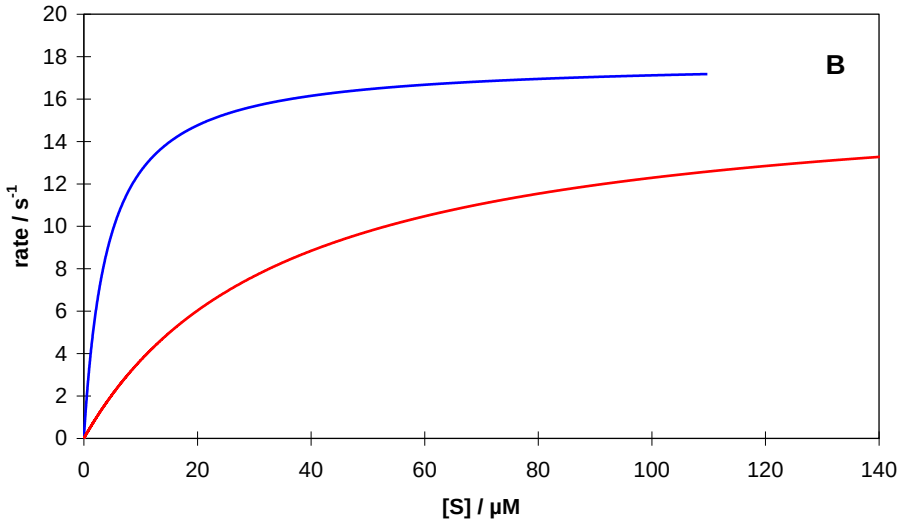
Blue: 10 μL 5.1 x 10^{-7} M trypsin injected into 950 μL 1.44 x 10^{-4}M BAEE

Red: plus 1.36 x 10^{-4} M benzamidine

(-) inhibitor: K_M = 4.17 μM; V_{max} = 0.091 μMol/s, k_{cat} = 17.8 s^{-1}

(+) inhibitor: K_M = 35.1 μM; V_{max} = 5.9 x 10^{-4} μMol/s, k_{cat} = 0.11 s^{-1}, K_i = 18.4 μM

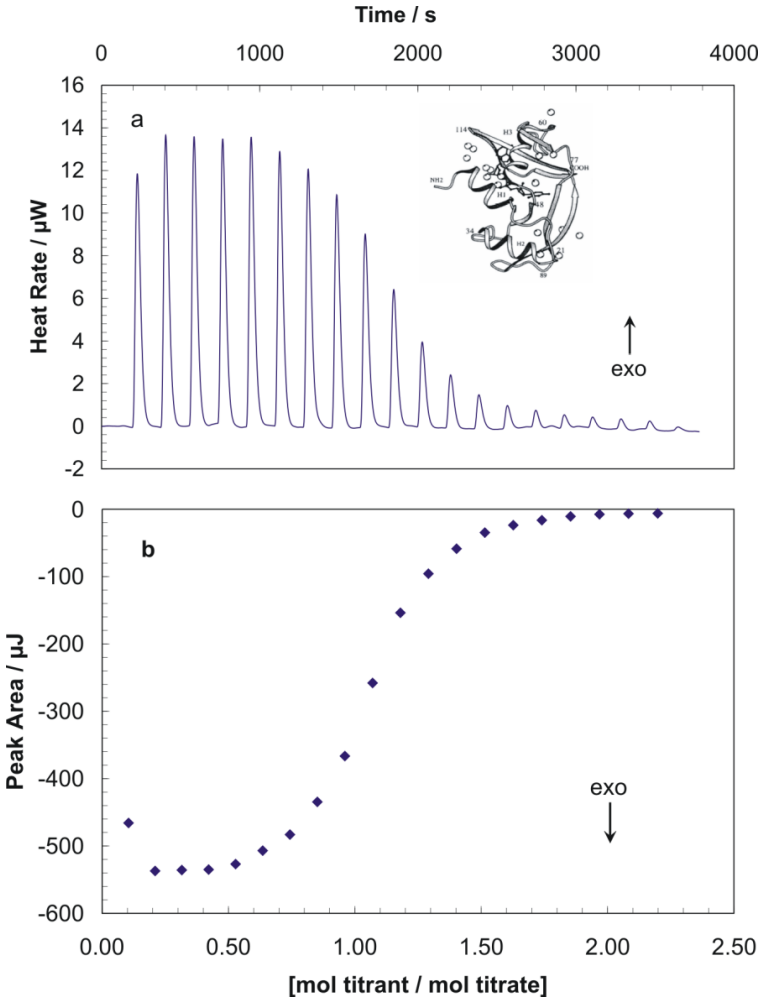




Techniques

Technique 1: Broadening the Binding Constant Range

2'-CMP titrated into RNase A



The shape of the binding curve determines the accuracy of K_a and ΔH.

What if K_a is outside 10^3 – 10^8 M^{-1}?

Technique 1: Broadening the Binding Constant Range: Weak Affinity

Five Tips for fitting data in which $K_{ITC}n[M]_{cell} < 1$ or $K_a < 10^3$

1) The reaction must be carried out to a molar ratio well past the reaction stoichiometry.
   Reload injection syringe and append the two sets of data.

2) Ligand and macromolecule concentrations must be accurately known.

3) Reaction stoichiometry (or enthalpy) must be known.

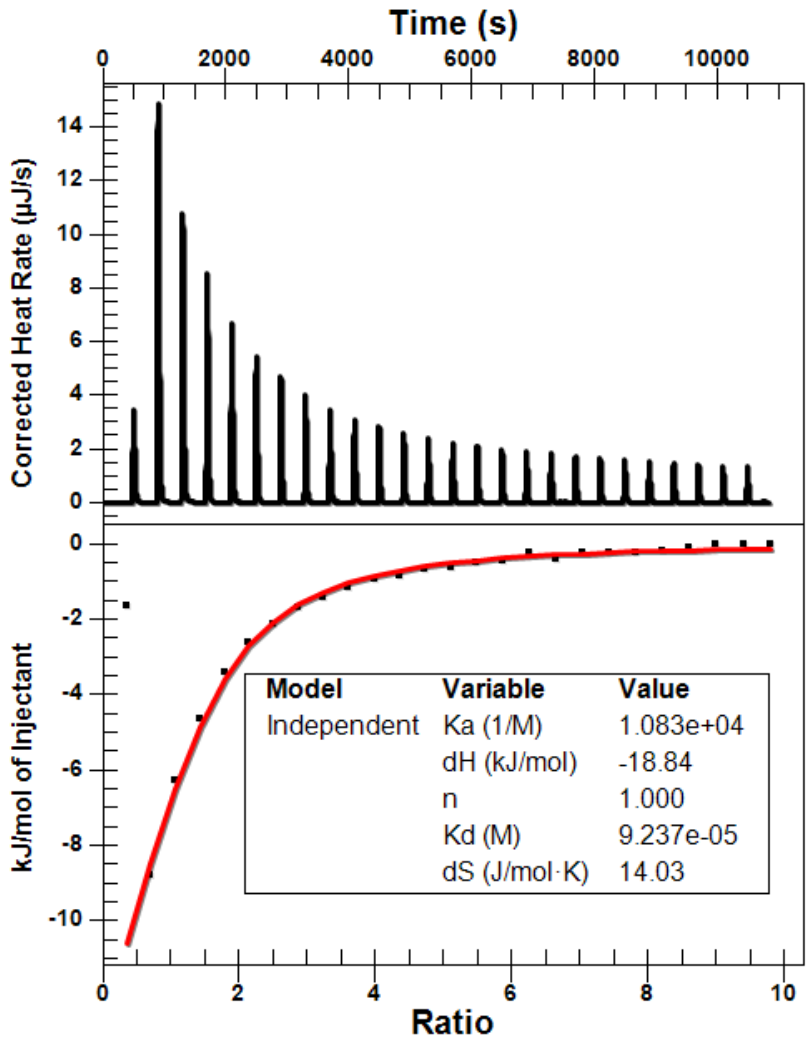
4) The experiment has adequate signal to noise.

5) Perform titration so that final stoichiometry is different
   Fitting algorithms should converge upon one solution – try Universal Fit.

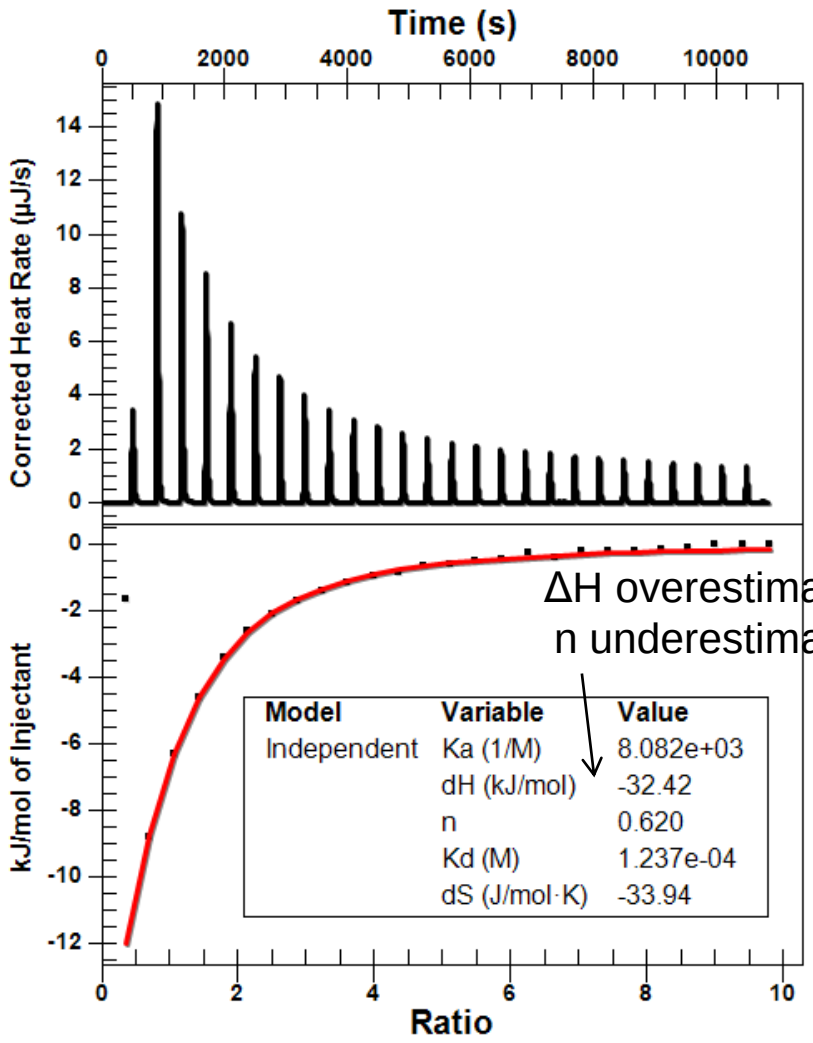
Turnbull, W. B. and Daranas, A. H. (2003) On the Value of c: Can Low Affinity Systems be Studied by Isothermal Titration Calorimetry? *J. Am. Chem. Soc. 125,*14859-14866.

Weak Binding - Data fitting Techniques

Fixed “n”

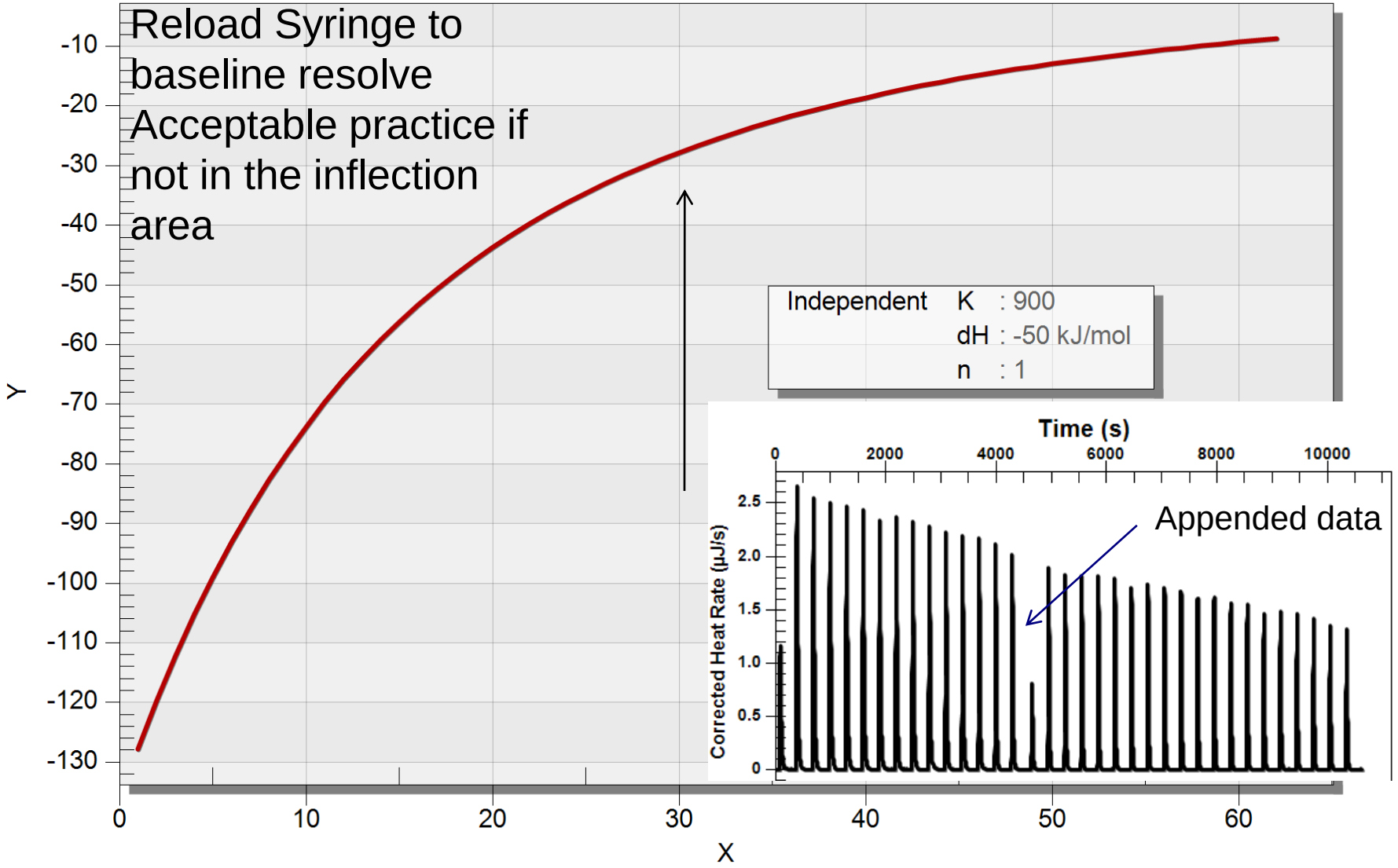


Floating “n”



ΔH overestimated
n underestimated

Low Ka
Modeling data for 4 mM titrant → 100 µM titrand



Technique 1: Broadening the Binding Constant Range: Weak or Strong Affinity

- Modeling data is Important
- Concentration determination

Competition Technique for Weak Binding

5’-CMP titrated into RNase A

Setup

Experiment 1 (Red)

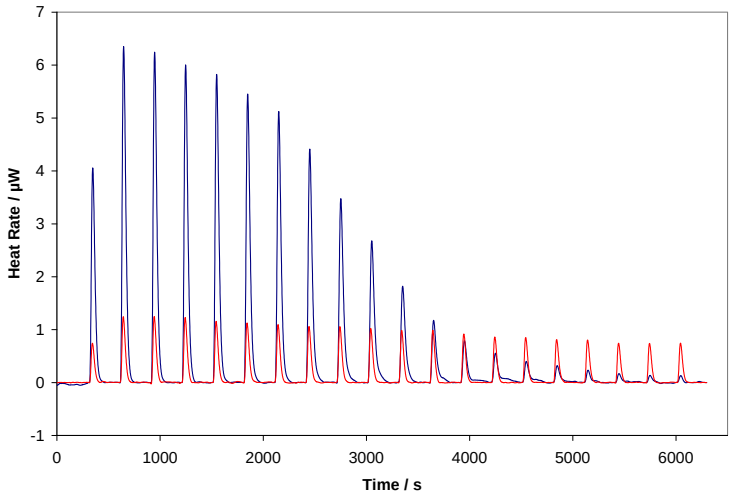
- 1.3 mM 5’CMP (titrant)
- 70 µM Rnase A (titrand)
- 20 × 5 mL injections at 25 °C

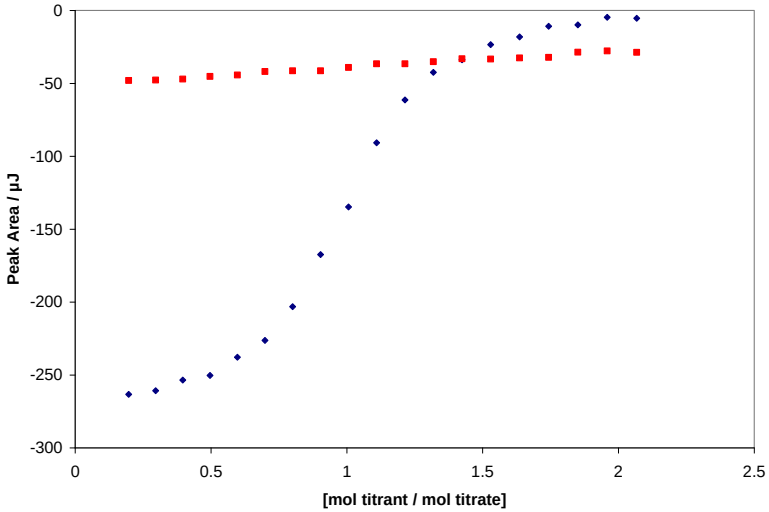
Experiment 2 (Blue)

- 1.3mM 2’CMP (titrant)
- 0.32 µM 5’CMP and 70 µM Rnase A (titrand)

Results – Competition experiment

- n = 1
- K_a = 3.1 x 10^3 M^{-1}
- Enthalpy of binding: -47 kJ/mol

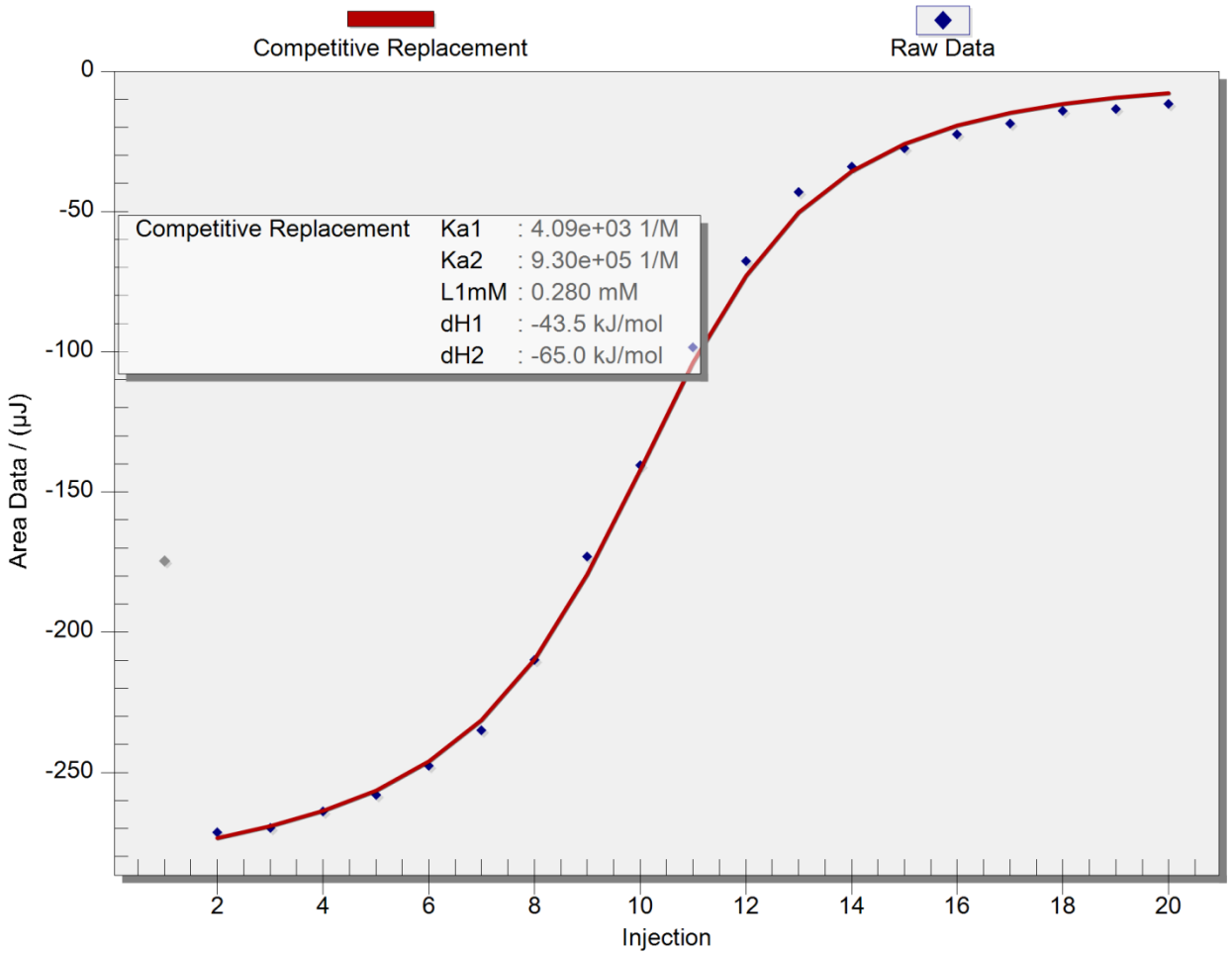




Measure affinity of very tight binding of molecules

Competition: Weak Affinity

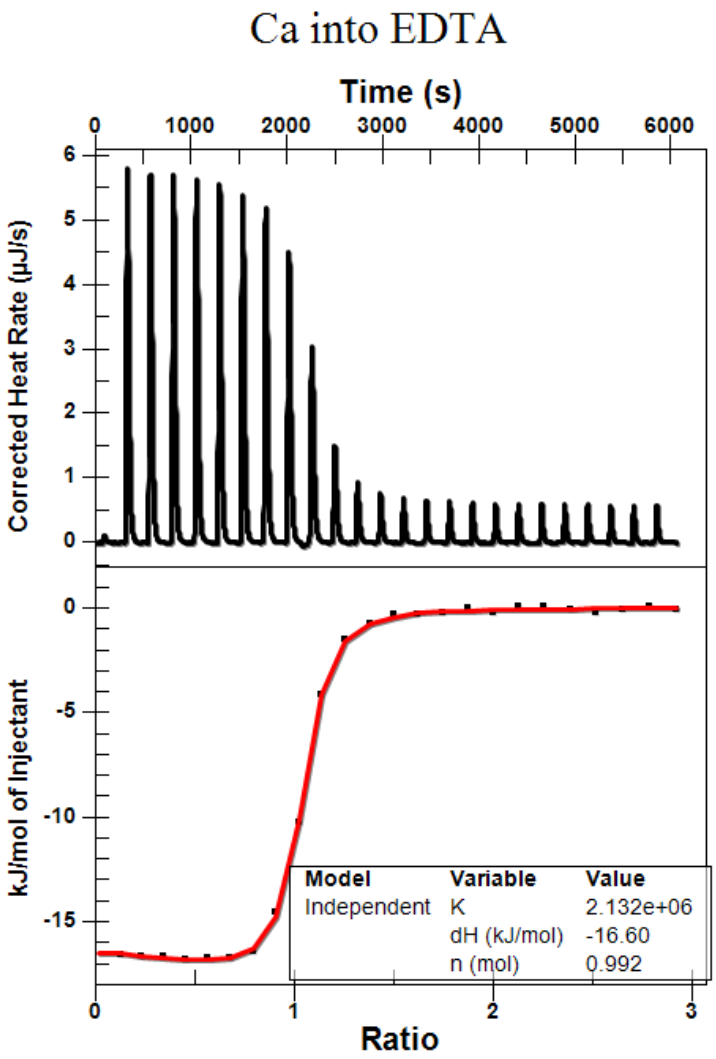
1.3 mM 2'CMP → 70 uM Rnase + 0.28 mM 5'CMP

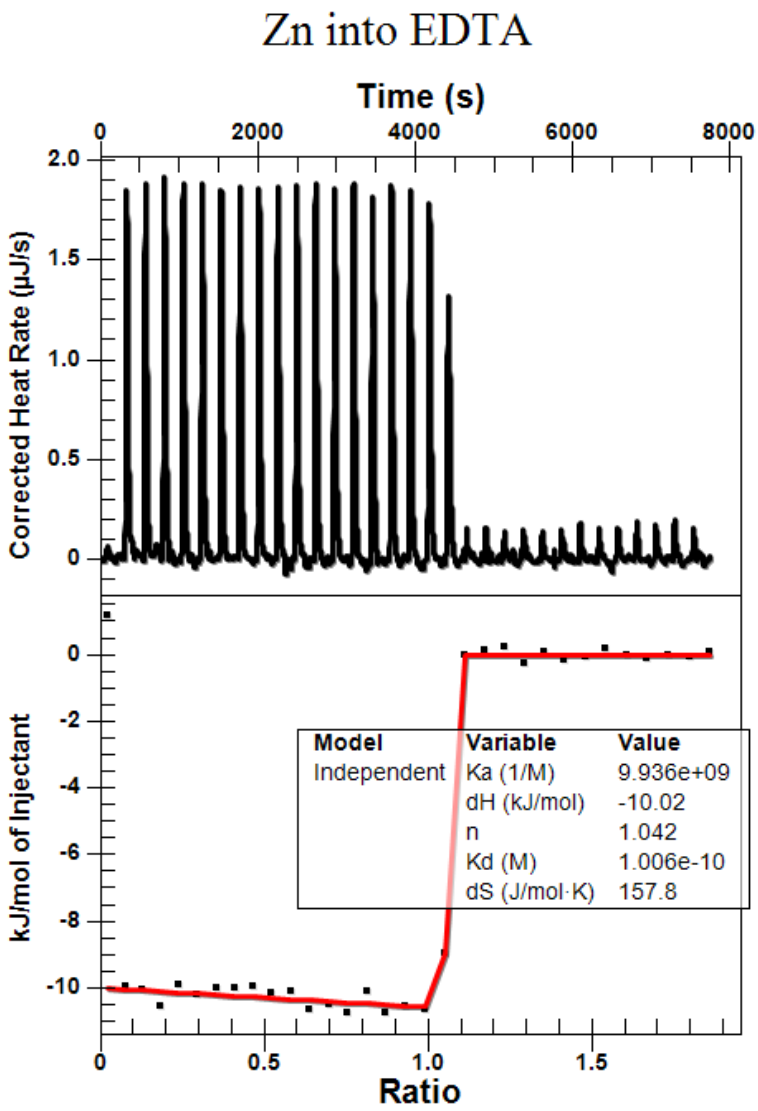


Variables

Name	Value
Ka1 (1/M)	4.09e+03
Ka2 (1/M)	9.30e+05
L1mM (mM)	0.280
dH1 (kJ/mol)	-43.5
dH2 (kJ/mol)	-65.0

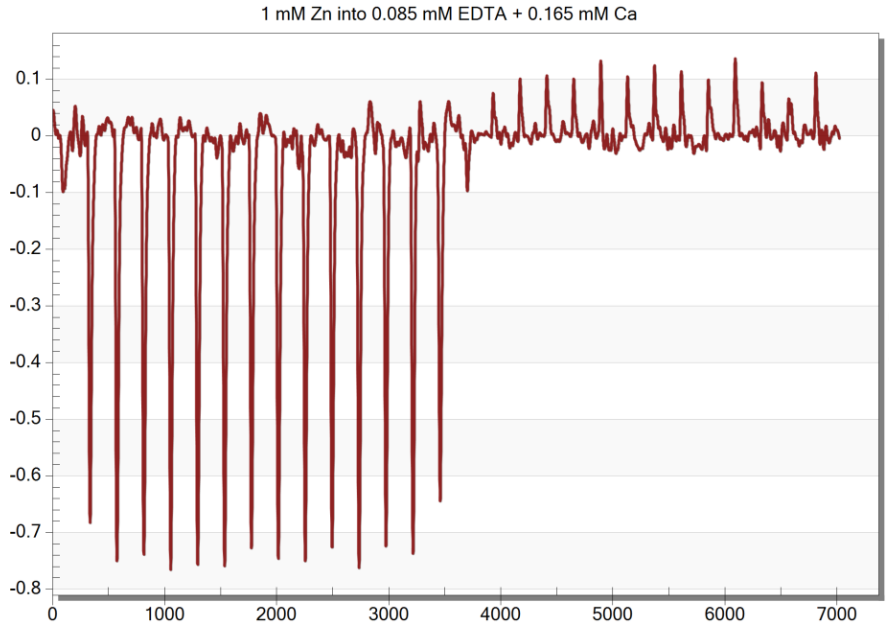
Competition Technique for Tight Binding

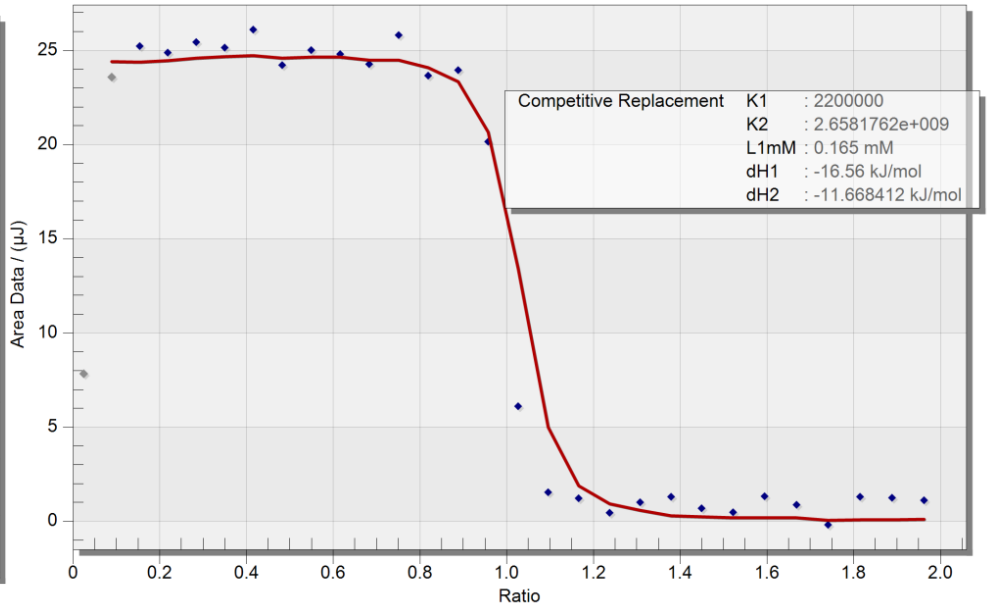




Competition: High Affinity

1 mM Zn^{2+} titrated into 85 µM EDTA + 165 µM Ca^{2+}



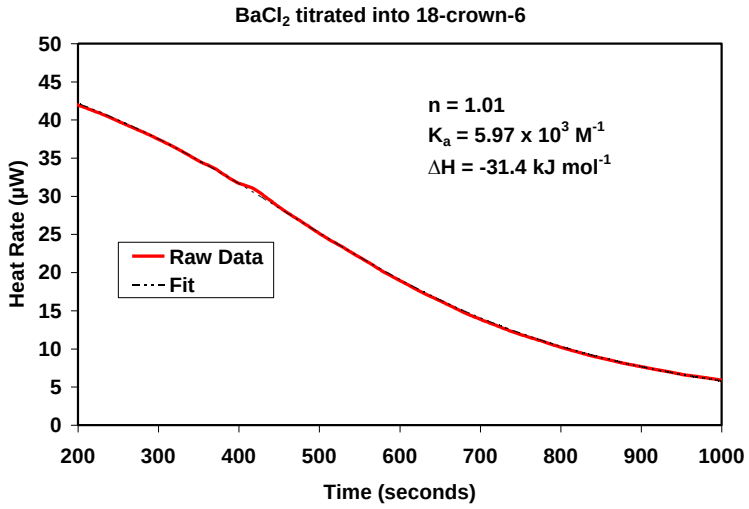


Variables

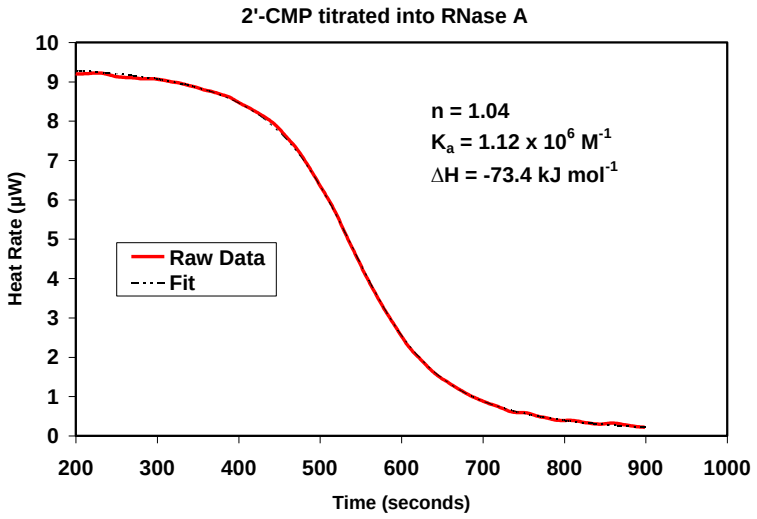
Name	Value	Vary
K1	2.30e+06	
K2	3.20e+09	✓
L1mM (mM)	0.165	
dH1 (kJ/mol)	-16.6	
dH2 (kJ/mol)	-11.7	✓

Continuous titration & binding

- **Typical ITC experiment**
  - 20 - 30 data points
  - 1 - 2 hours (SV) & 0.5 to 1 hour (LV)

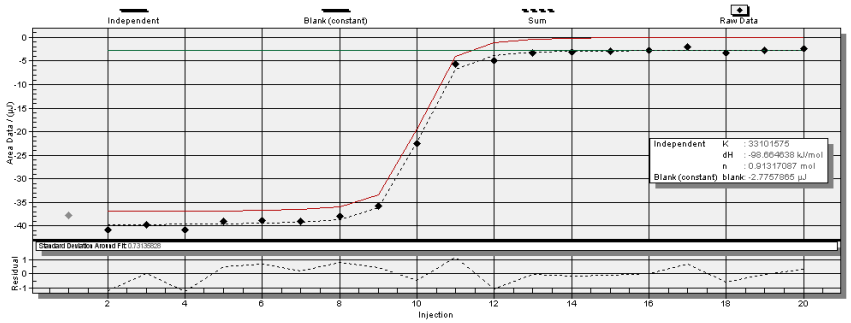


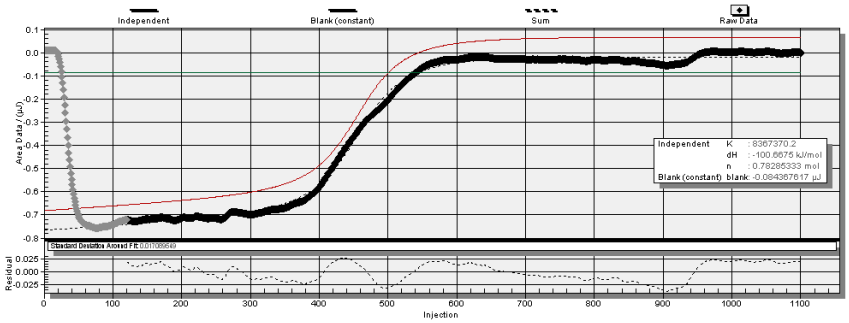
- **Continuous ITC experiment**
  - > thousand data points
  - 8 - 20 minutes (0.05 - 0.15 mL titrant/sec)
  - More data points at inflexion of binding isotherm

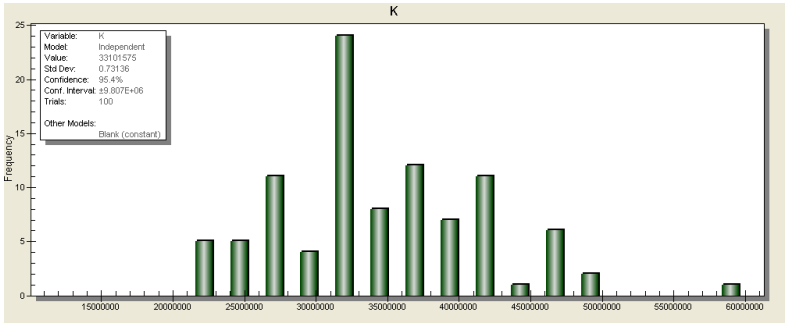


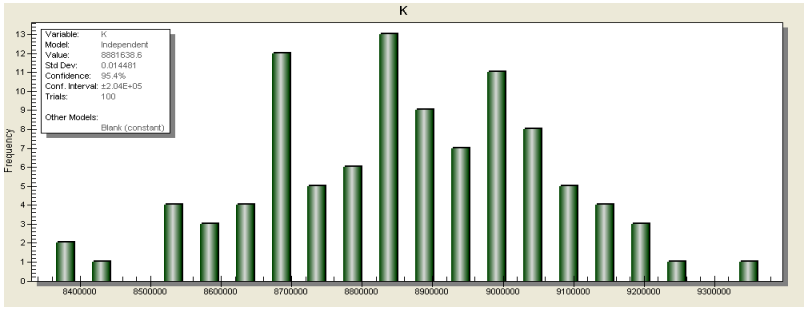
Incremental vs. Continuous titration

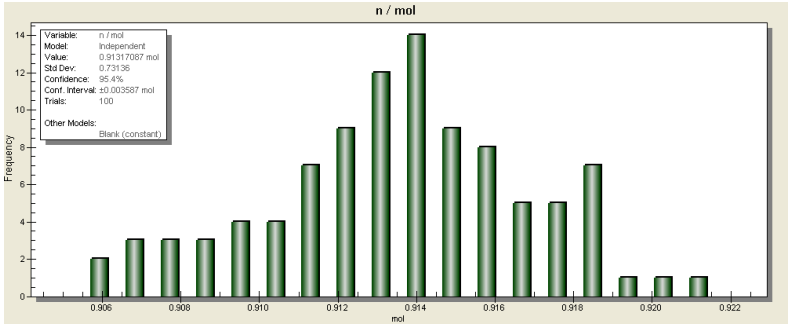
Incremental titration vs Continuous titration

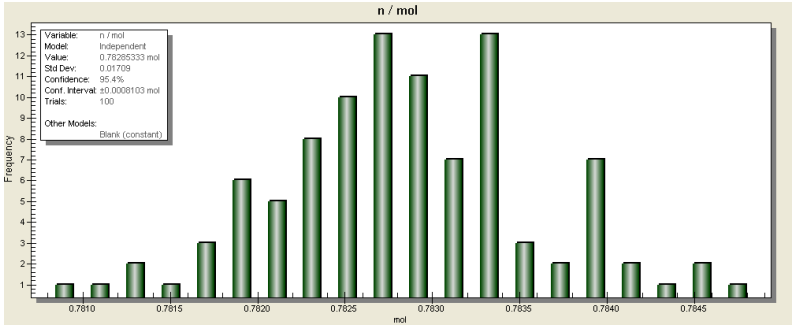












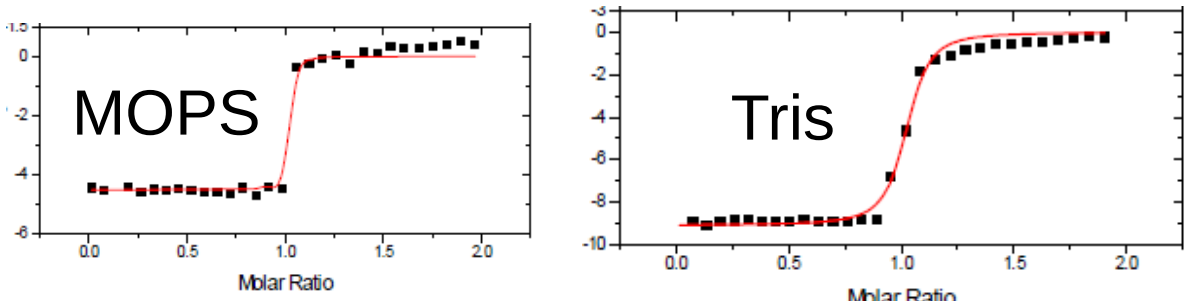
Technique 1 Recap: Broadening the Binding Constant Range: Weak or Strong Affinity

1. Use the continuous titration to increase the reliability of strong K_a

2. Know concentrations for weak K_a and append data sets

3. Competition
   - Special consideration when determining appropriate concentrations

4. Change conditions: buffer, pH, temperature



1 mM Zn → 0.10 mM EDTA
100 mM Buffer pH 7.4

Technique 2: Temperature Variation

- Two Effects to Investigate:

1. Solvation
2. Heat capacity determination
   - $\Delta C_p = (\partial \Delta H / \partial T)_p$

Technique 2: Temperature Variation

Vary the temperature.

Uncover the temperature dependence of ΔH.

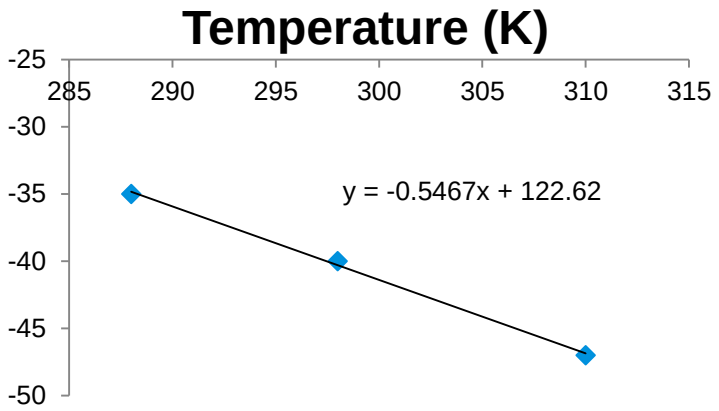
- This dependence is related to the macromolecule's hydrophobic interactions more specifically, solvation.
- If ΔH is more exothermic at a higher temperature, it is believed that hydrophobic bonds are formed.
- If ΔH is less exothermic at the higher temperature, it is considered that hydrophobic bonds are broken.

Technique 2: Temperature Effects, Heat Capacity

ΔCp is also related to enthalpy and entropy

$$\Delta Cp = \left(\frac{\partial \Delta H}{\partial T}\right)_p$$

$$\Delta C_p = T\frac{\delta \Delta S}{\delta T}$$



Δ Cp in biological reactions involves changes that affect ordered water
- Δ Cp indicates a decrease in the amount of ordered water
+ Δ Cp increase in amount of ordered water (PNAS (1977), 74, 2236-2240)

In case of L binding to P, if Δ Cp <0, this is associated with ligand-induced structural changes that bury hydrophobic resides and liberate solvent (JACS (1964), 86, 4302)

Technique 3: Utilizing the Reverse Titration

Unexpected stoichiometry, Unexpected enthalpy or unsure of concentration?

Before proceeding with developing a new mechanism, design an experiment in which the macromolecule is titrated into a solution of the ligand.

Forward:	*Reverse*
Ligand (L) → Macromolecule (M)	*M → L*

Obtaining meaningful ITC data: unexpected stoichiometry

Unexpectedly low stoichiometry could be due to:

- [receptor] lower than anticipated
- [ligand] higher than anticipated
- Receptor contains contaminating proteins
- Receptor is partially unfolded
- Multiple binding sites
- Wrong binding model
- Insufficient curvature in data: change concentrations
- Experimental Artifact

Technique 3: Utilizing the Reverse Titration: Reaction Stoichiometry is Unclear

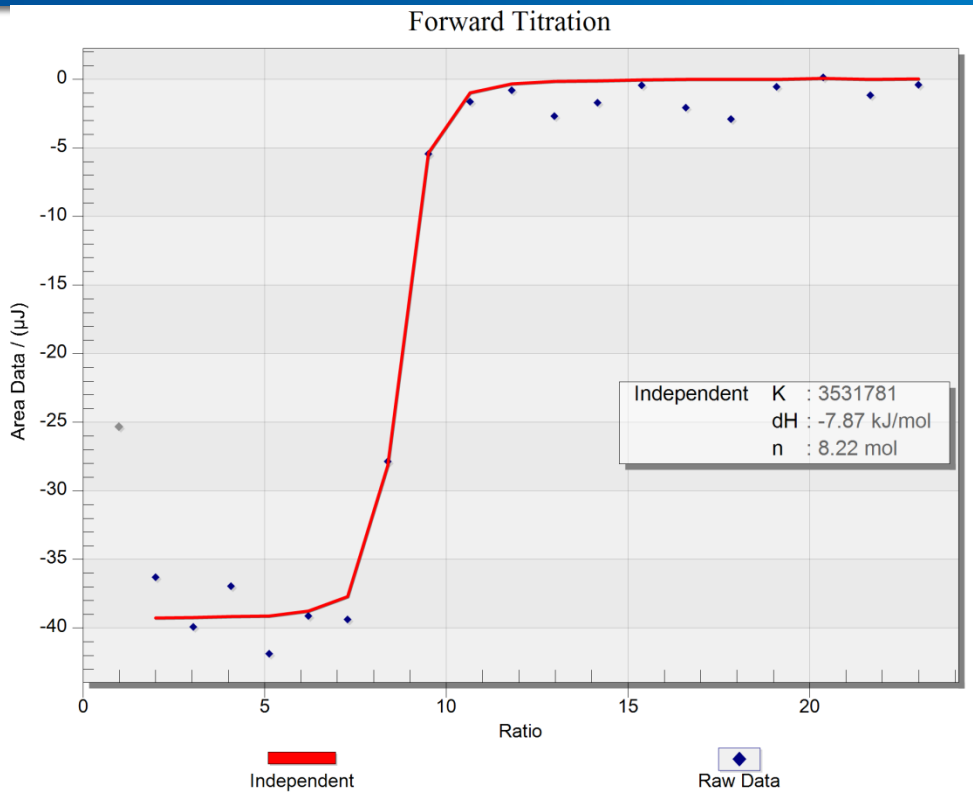
- Case 1: A→ B
- n = 0.7
- dH = 100

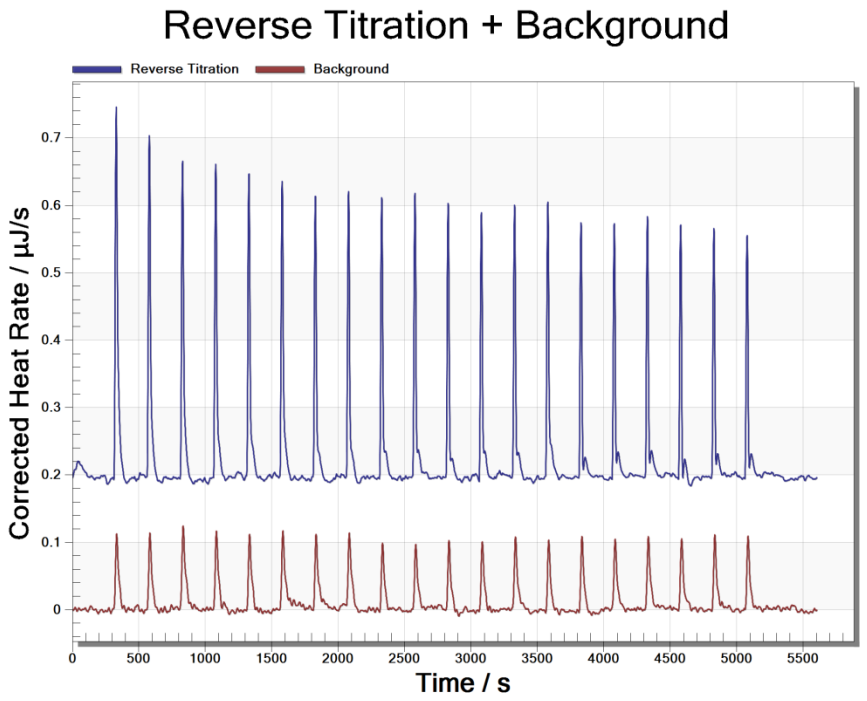
- Is the interaction really 1:1 or 1:2?

- Run a reverse titration (B→ A).
- If ΔH ~ 140 kJ/mol then it could be 1A:2B, [B] greater than expected
- If Δ H ~ 70 kJ/mol then it could be 1:1, [B] less than expected

For this relationship to be true, you must know the concentration of A.

Technique 3: Utilizing the Reverse Titration

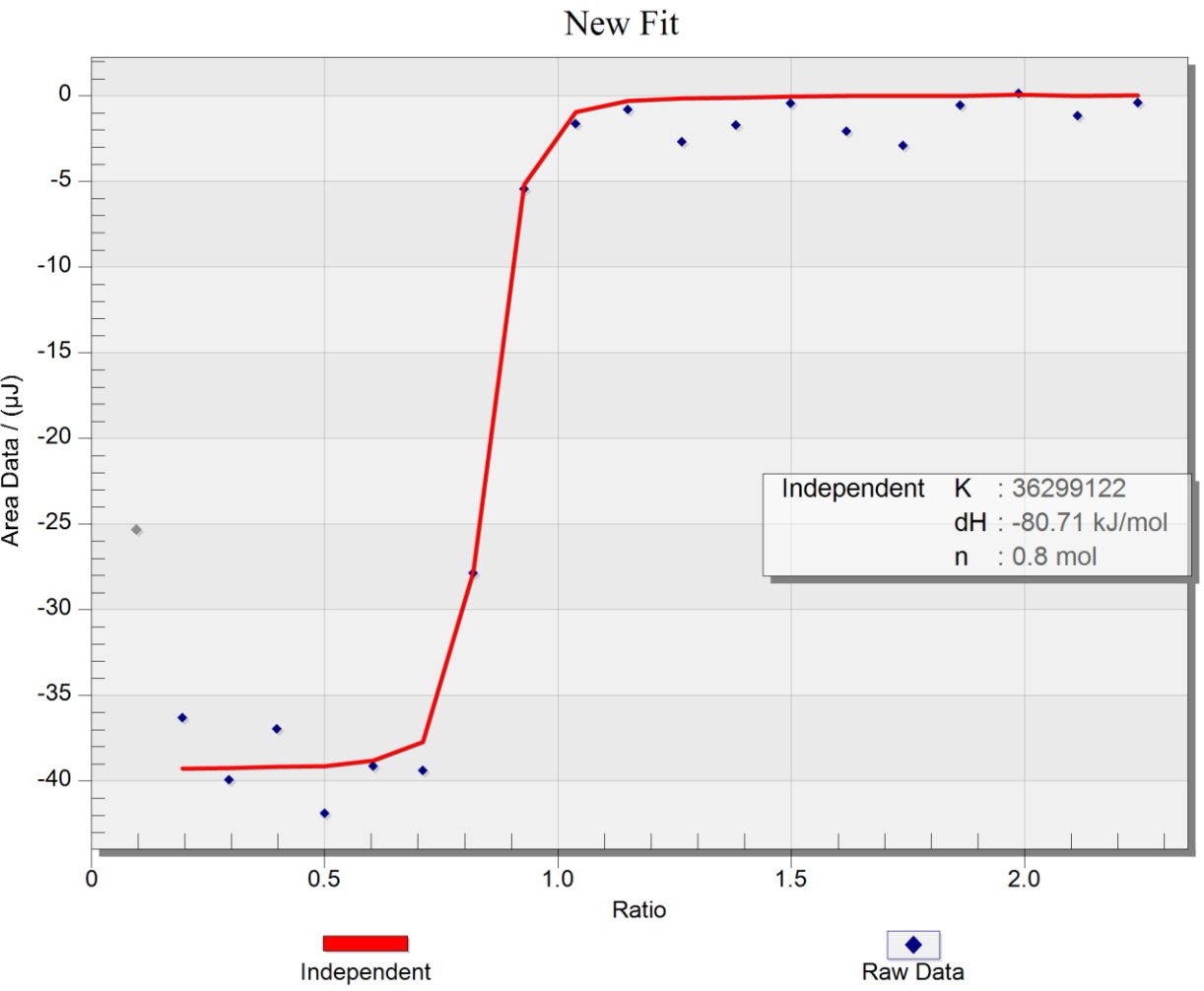




Forward Titration:
ΔH = -7.87 kJ/mol
n = 8.17 mol

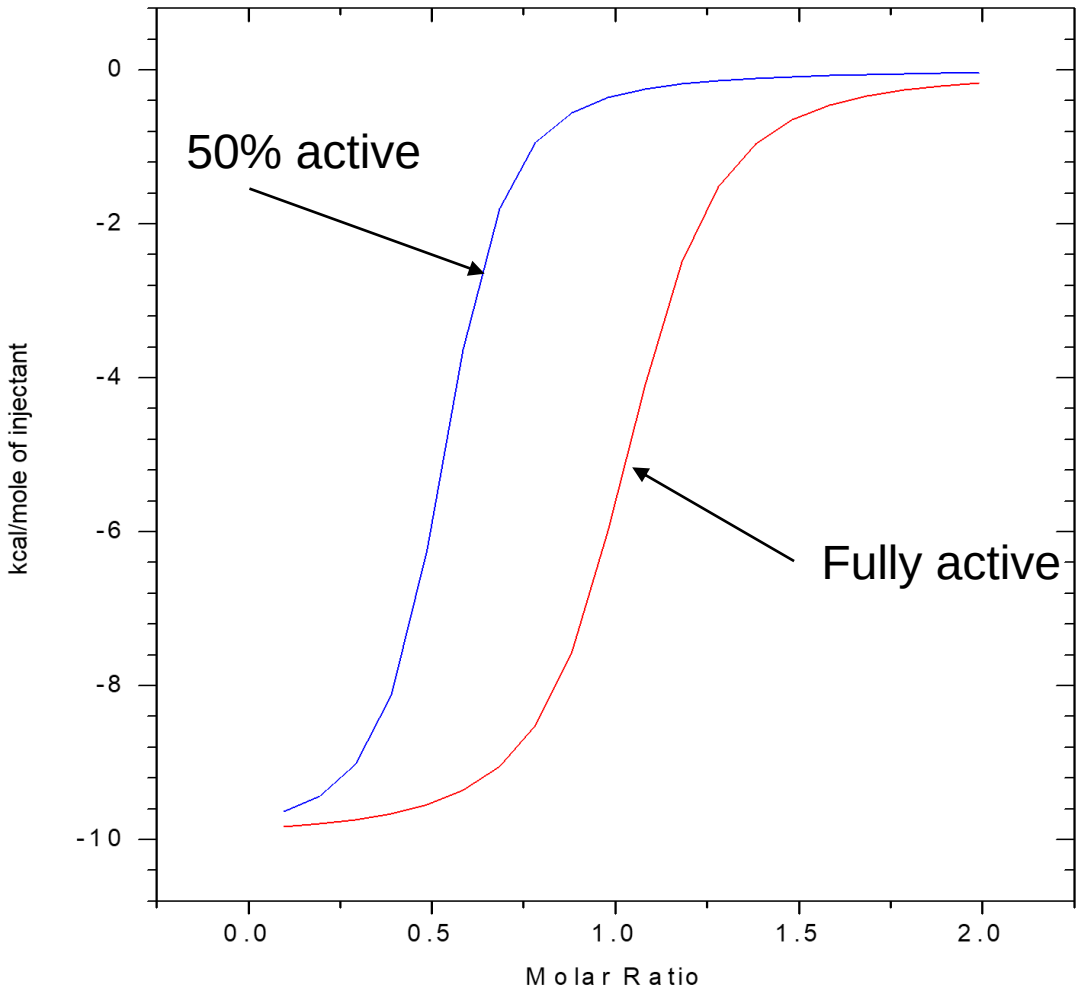
Reverse Titration: -9 µJ
Background: -2.96 µJ
ΔH = -80.4 kJ/mol
(-6 µJ/750 nmoles/injection)

Technique 3: Utilizing the Reverse Titration



The concentration of titrant was changed to have agreement between ΔH and unexpected stoichiometry is resolved!

Concern with Technique 3: Quality Control



- Protein Quality
  - Anti-quinidine antibody batches compared
  - Activity of antibodies immobilized on metal beads quantitatively measured

Technique 3: Utilizing the Reverse Titration: Resolution of Sequential Events

- Another important use of this method is when the forward titration contains multiple chemical events.

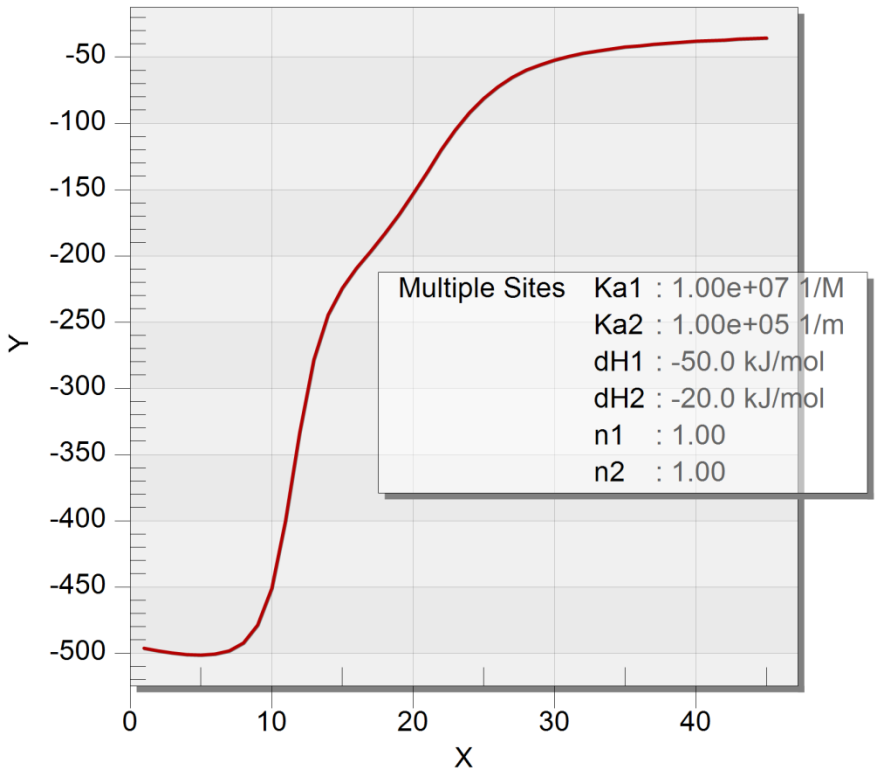
- Titrating ligand into the macromolecule leads to an initial chemical environment where the macromolecule is in excess and higher order complexes (LM_n) may form.

- If the overall binding constant, β, >> individual K values, it may not be possible to drive the concentration of ligand high enough to break up this complex.

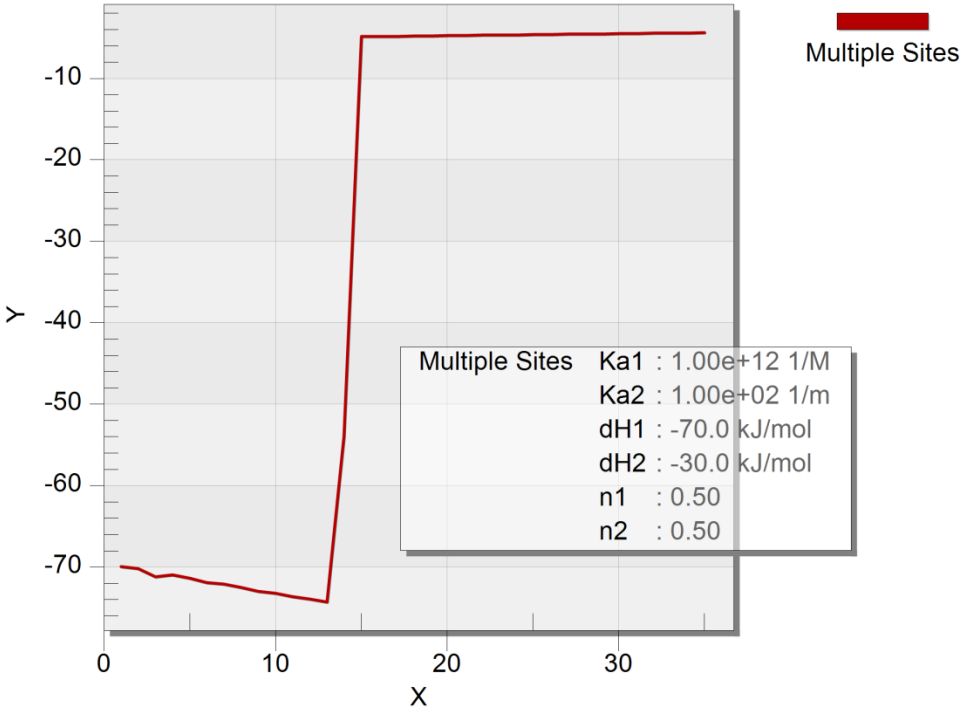
- Conducting the reverse titration (M → L), the ligand is originally in excess and sequential binding of macromolecule is expected
  - if $K_{n+1} >> K_n$, it is still possible that the individual species in the sequential process will not be observed.

Reverse Titration

M→L
Final M_2L



L→M



Here, K_{a1} is the β
K_{a2} is the dissociation of M_2L and formation of ML

Technique 3: Utilizing the Reverse Titration Recap

- The reverse titration can be used when the stoichiometry is unexpected or when the stoichiometry is well-defined.

Linked Equilibria

Origin of the heat

$$\Delta q_{\text{measured}} = \Delta q_{\text{binding}} + \Delta q_{\text{dilution}} + \Delta q_{\text{injection}} + \Delta q_{\text{other}}$$

Observed enthalpy of ligand-binding is given by:

No Proton Uptake → $\Delta H_{obs} = \Delta H_{int}$ (Buffer-independent)

Proton Uptake → $\Delta H_{obs} = \Delta H_{int} + \Delta m.\Delta H_{ion}$ (Buffer-dependent)

where ΔH_{obs} = Observed enthalpy
ΔH_{ion} = Intrinsic enthalpy due solely to ligand binding
ΔH_{ion} = Ionization enthalpy of the reaction buffer
Δm = Number of protons exchanged

Technique 4: Quantifying Protons Released

Data analysis begins by listing the components that make up the overall binding enthalpy, ΔH_{ITC}, which comes from fitting the data (equation 1).

$$mMB + nHL \rightleftarrows mML + nHB \qquad \textbf{[equation 1]}$$

Reaction	Enthalpic Designation
$mM + L^{-} \rightleftarrows mML$	$m\,\Delta H_{ML},\ K_{ML}^{\ m}$
$mMB \rightleftarrows mM + B$	$-m\Delta H_{MB},\ K_{MB}^{\ -m}$
$n(H + B \rightleftarrows HB)$	$n\Delta H_{HB},\ K_{HB}^{\ n}$
$H_nL \rightleftarrows L + nH$	$-n\Delta H_{HL},\ K_{HL}^{\ -n}$

Scheme 1. Set of coupled reactions for a metal (M) binding a ligand (L); *m* is the number of metals that bind the ligand and *n* is the number of protons that are released for this binding process.

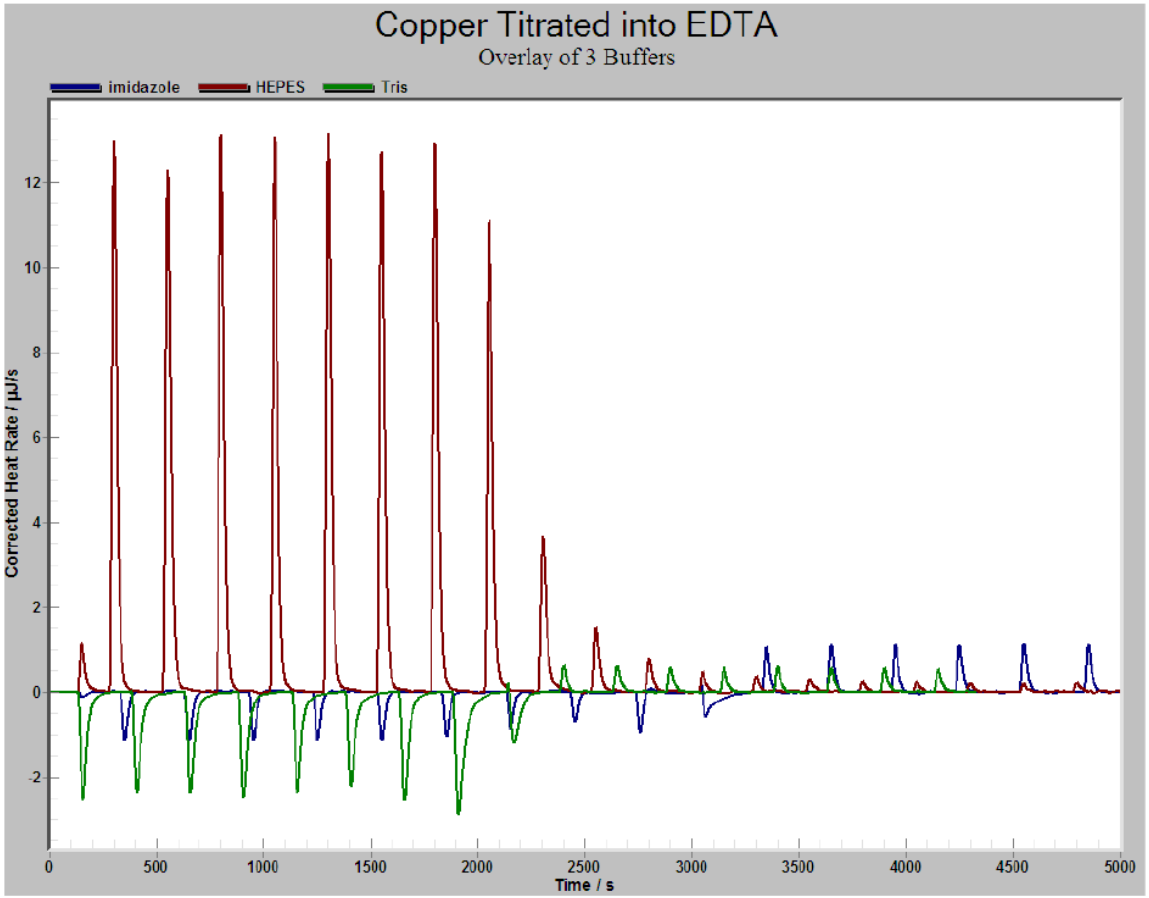
Technique 4: Quantifying Protons Released

$$\Delta H_{ITC} = \Delta H_{M-EDTA} + n_3 \Delta H_{H-Buffer} - \Delta H_{M-Buffer} - n_1 \Delta H_{H-EDTA} - n_2 \Delta H_{H_2-EDTA}$$

Protons released, n_1, n_2 and n_3, take into consideration the percentage of each species in solution, where $n_3 = n_1 + n_2$, which depends on the pK_a's of EDTA.

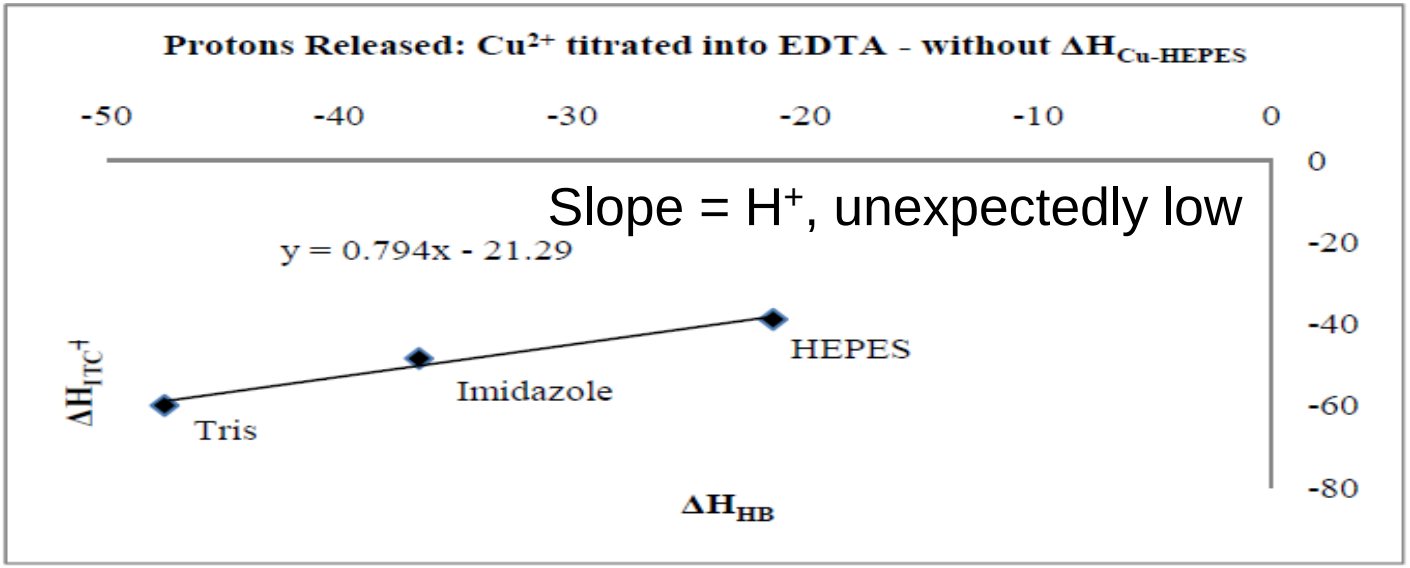
Reaction	*n*	*ΔH (kJ/mol)*
$Cu^{2+} + EDTA^{4-} \rightleftarrows CuEDTA^{2-}$	*1*	*-34 (5)*
$EDTAH^{3-} \rightleftarrows EDTA^{4-} + H^{+}$	*0.95*	*-23 (5)*
$EDTAH_2^{2-} \rightleftarrows EDTA^{4-} + 2H^{+}$	*0.05*	*-40 (5)*
$CuBuffer^{2+} \rightleftarrows Cu^{2+} + Buffer$	*1*	*?*
$HBuffer^{+} \rightleftarrows H^{+} + Buffer$	*1.05*	*NIST(5)*

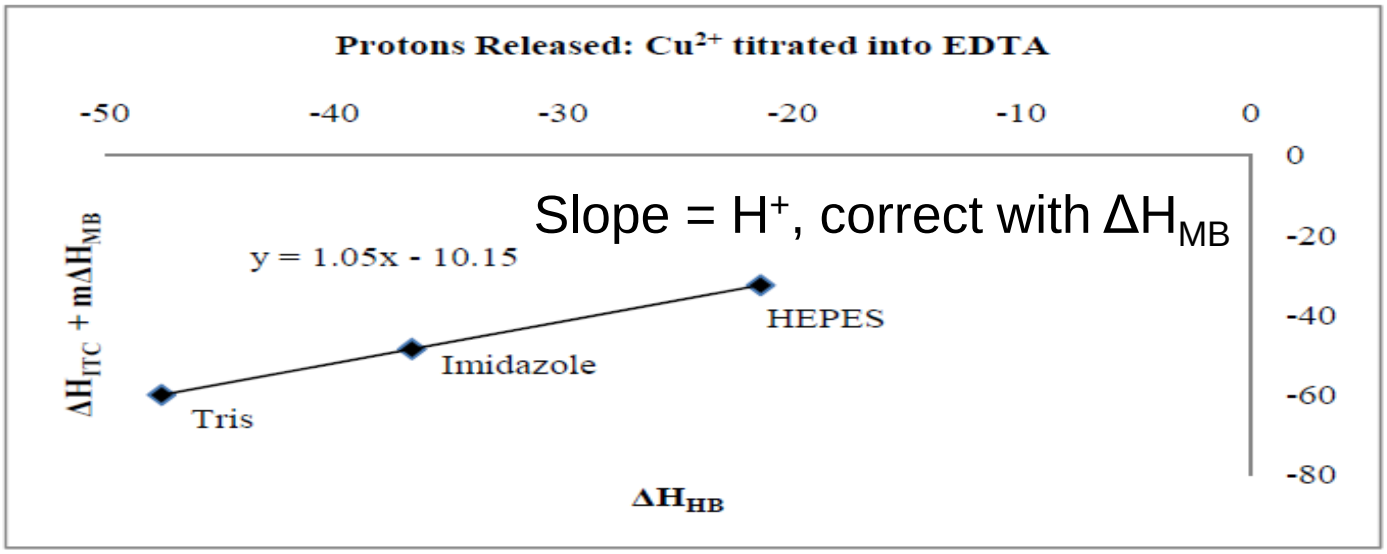
Technique 4: Quantifying Protons Released



$$\Delta H_{ITC} + \Delta H_{M-Buffer} = n_3 * \Delta H_{H-Buffer} + (\Delta H_{M-EDTA}\ n_1 * \Delta H_{H-EDTA} - n_2 * \Delta H_{H_2-EDTA})$$

Technique 4: Quantifying Protons Released:

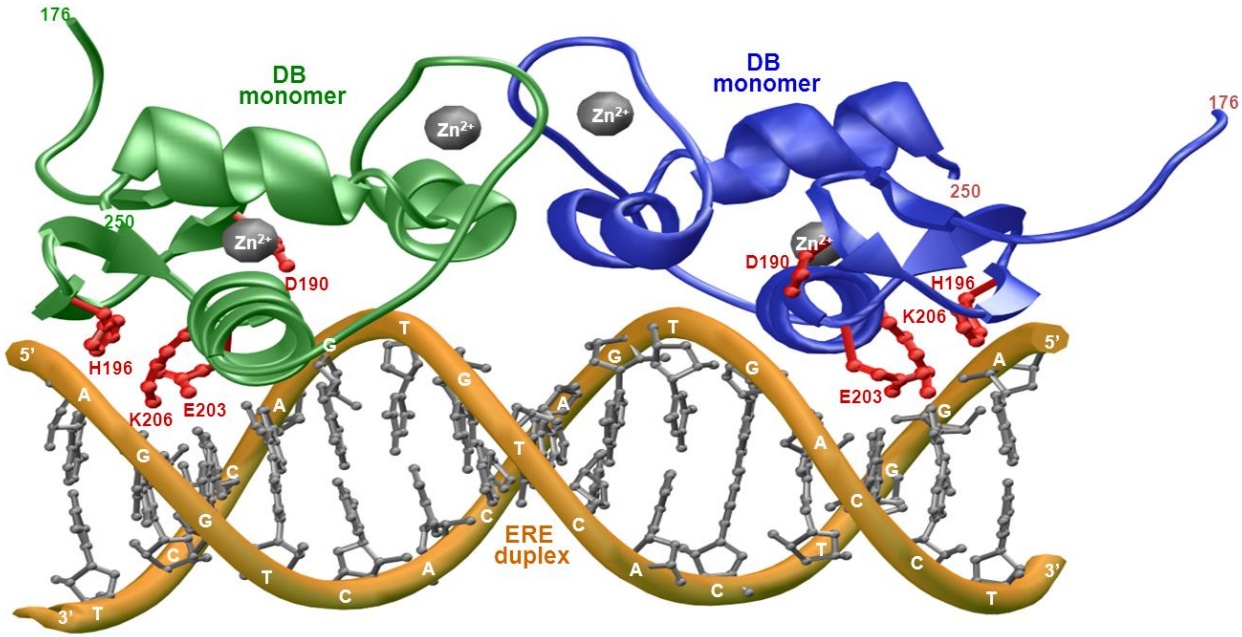




Technique 4: Quantifying Protons Released

- For many systems plotting $\Delta H_{H\text{-}Buffer}$ v. ΔH_{ITC} will provide the number of protons released.
  - If the ligand has additional chemistry with the buffer, then this needs to be considered.
- Systems with secondary chemistry occurring require more consideration.
- In order to determine which method is correct for your system, write down all possible chemistry occurring in the reaction cell.

Technique 5: Buffer Independency and Linked Equilibria



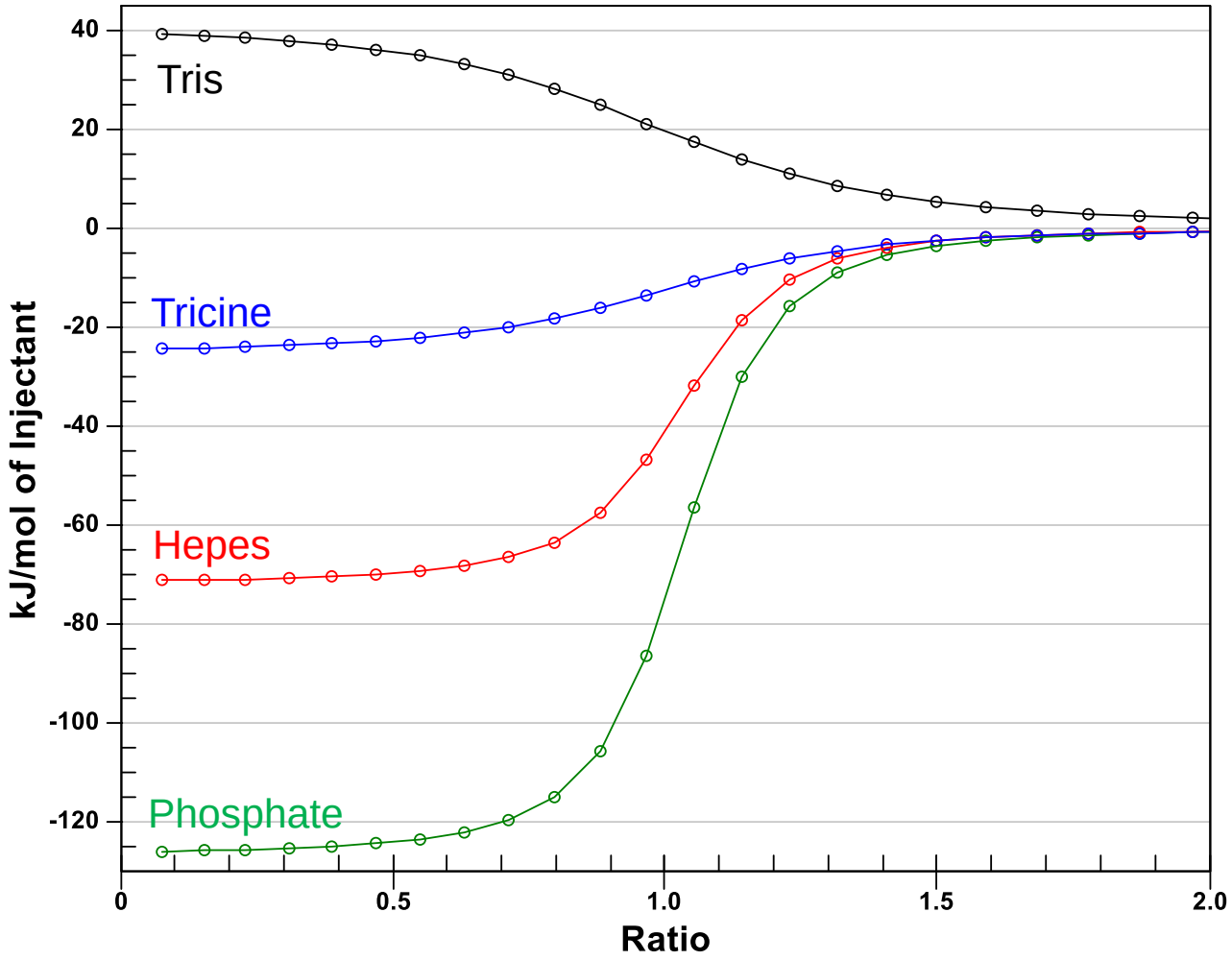
Binding of ERα to DNA is coupled to proton uptake at H196 and E203

Macromolecular interactions are often coupled to linked equilibria such as:

(1) Folding/Unfolding
(2) Oligomerization/Dissociation
(3) Counterion release
(4) Protonation

Technique 5: Buffer Independent Enthalpic Values and Protons Released

Binding of ERα to DNA in various buffers analyzed by ITC:

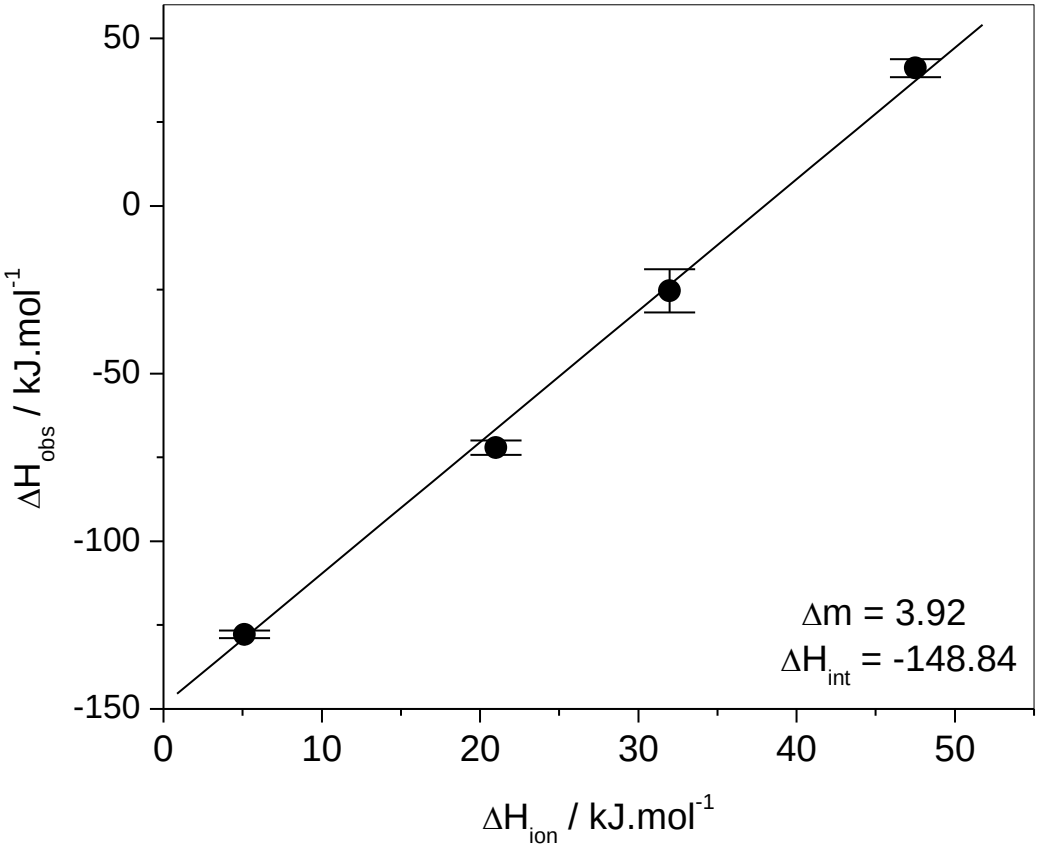


Technique 5: Buffer Independent Enthalpic Values and Protons Released

Binding of ERα to DNA in various buffers analyzed by ITC:

Binding enthalpy is given by:
$\Delta H_{obs} = \Delta H_{int} + \Delta m.\Delta H_{ion}$

A plot of ΔH_{obs} vs ΔH_{ion} is a straight line:
Slope = Δm
Y-intercept = ΔH_{int}



Technique 6: ITC and Linked Equilibria (Counterion Release)

Electrostatically charged macromolecules such as DNA do not “swim” freely in solution but the negatively charged phosphate backbones are tightly shielded with cations so as to neutralize the net charge close to zero

Such cations are referred to as “counterions” → Green spheres

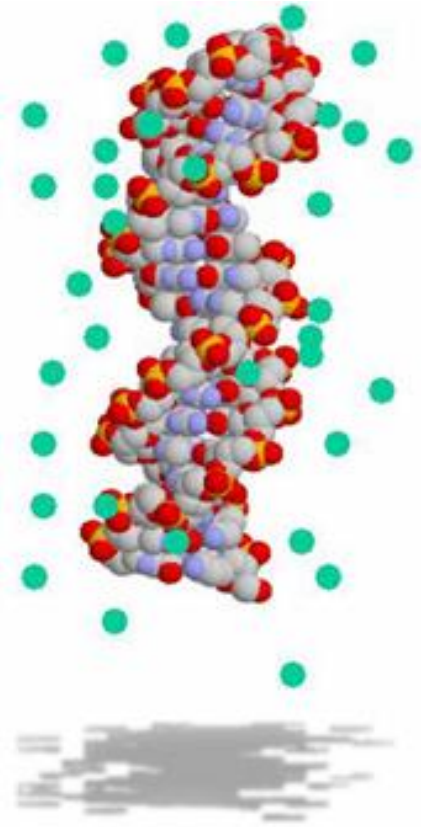
Upon binding to a target protein, the counterions are displaced from the DNA backbone in an entropically-favorable manner

Such release of counterions provides a major contribution to the free energy (ΔG) associated with protein-DNA interactions

How can we determine the contribution due to the counterion displacement (ΔG_{ion}) to the overall ΔG for protein-DNA interactions?

Knowledge of ΔG_{ion} sheds key thermodynamic insights into the role of ionic interactions driving protein-DNA interactions

Additionally, comparison of ΔG_{ion} for the binding of a transcription factor to different gene promoters may provide insights into the differential binding at atomic level

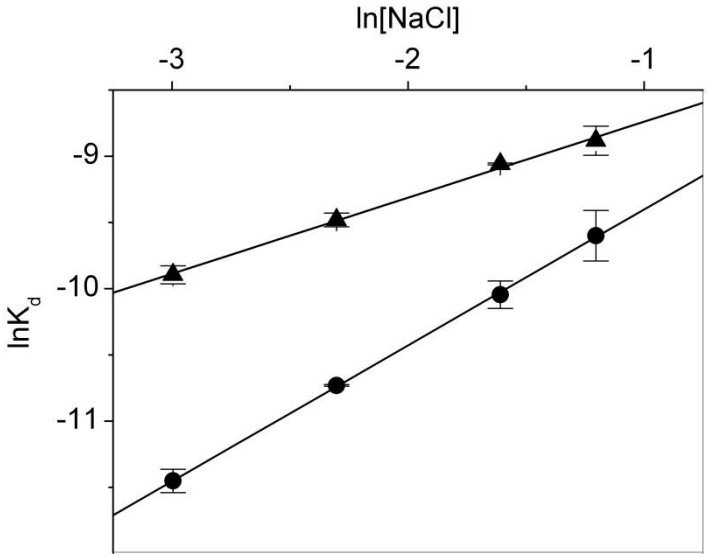


Technique 6: ITC and Linked Equilibria (Counterion Release)

Free energy change (ΔG) upon ligand binding can be dissected into two major constituent components by the following relationship:

$$\Delta G = \Delta G_{lig} + \Delta G_{ion} = RT\ln K_d$$

where ΔG_{lig} is the contribution due to direct ligand binding and ΔG_{ion} is the contribution due to the indirect displacement of counterions upon ligand binding



ΔG_{ion} at a given NaCl concentration can be calculated from the following relationship based on the polyelectrolyte theory:

$$\Delta G_{ion} = \psi RT\ln[NaCl]$$

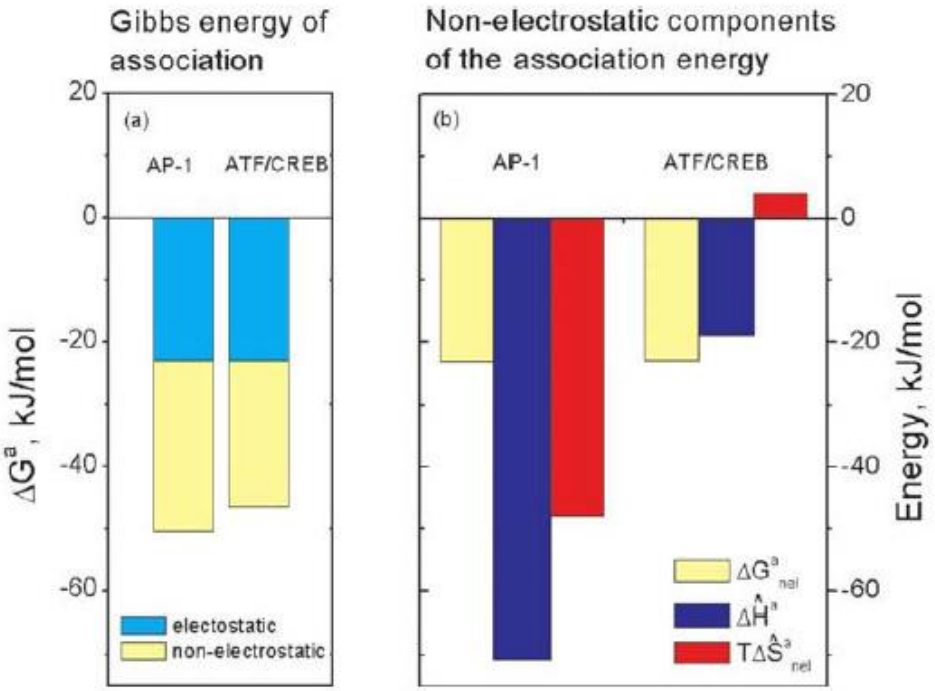
where ψ is the fractional degree of net counterions displaced upon ligand binding

ψ can be calculated from the slope of a plot of $\ln K_d$ versus ln[NaCl] assuming the following linear relationship based on thermodynamic linkage:

$$\ln K_d = \psi \ln[NaCl] + \ln K_0$$

where ψ and $\ln K_0$ are respectively the slope and the y-intercept of $\ln K_d$-ln[NaCl] plot

GCN4-bZIP Binding to DNA



- **Specificity of bZIP is due to the non-electrostatic component of the binding energy**
- **Electrostatic component is purely entropic**
- **Nonelectrostatic component has enthalpic and entropic contributions:**
  - **Changes in conformation**
  - **Dehydration**
  - **Direct protein-DNA interactions**

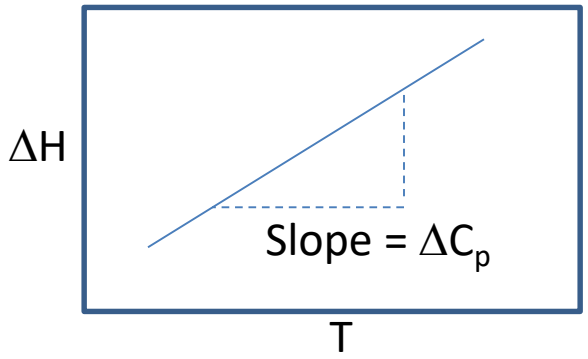
* Peter L. Privalov and Anatoly I. Dragan, 2006

Technique 6: ITC and Linked Equilibria (Heat Capacity Change)

Heat capacity change (ΔC_p) is a key thermodynamic parameter associated with macromolecular interactions

ΔC_p is defined as change in enthalpy (ΔH) per unit change in temperature (T) as expressed by Kirchhoff's law:

$$\Delta C_p = d(\Delta H)/dT$$



Consider the following observation:

$$C_{p\ (water)} = 4 \times C_{p\ (aluminum)}$$

In other words, heat needed to raise the temperature of water by 1°C is four times that needed for aluminum (assuming equimolar quantities of both substances)

Clearly, ΔC_p is telling us something about the differential properties of molecular structure of these substances

For macromolecular interactions, ΔC_p is typically determined from the slope a plot of ΔH versus T (over the temperature range 15-35°C)

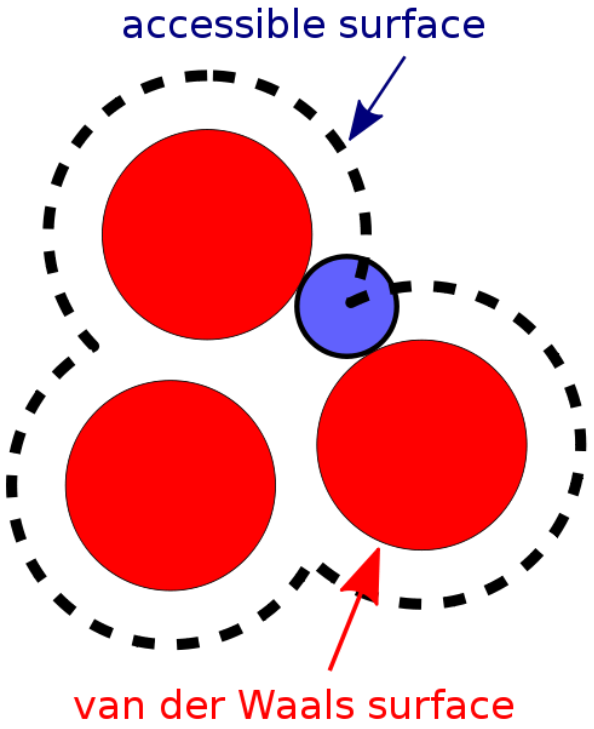
Technique 6: ITC and Linked Equilibria (Heat Capacity Change)

Changes in the solvent accessible surface area (SASA) are often associated with macromolecular interactions

Knowledge of such change in SASA (ΔSASA) can provide important structural insights into macromolecular interactions

A small value of ΔSASA may be indicative of two macromolecules coming together in a rigid-body fashion

A large value of ΔSASA may be indicative of structural or conformational changes associated with macromolecules



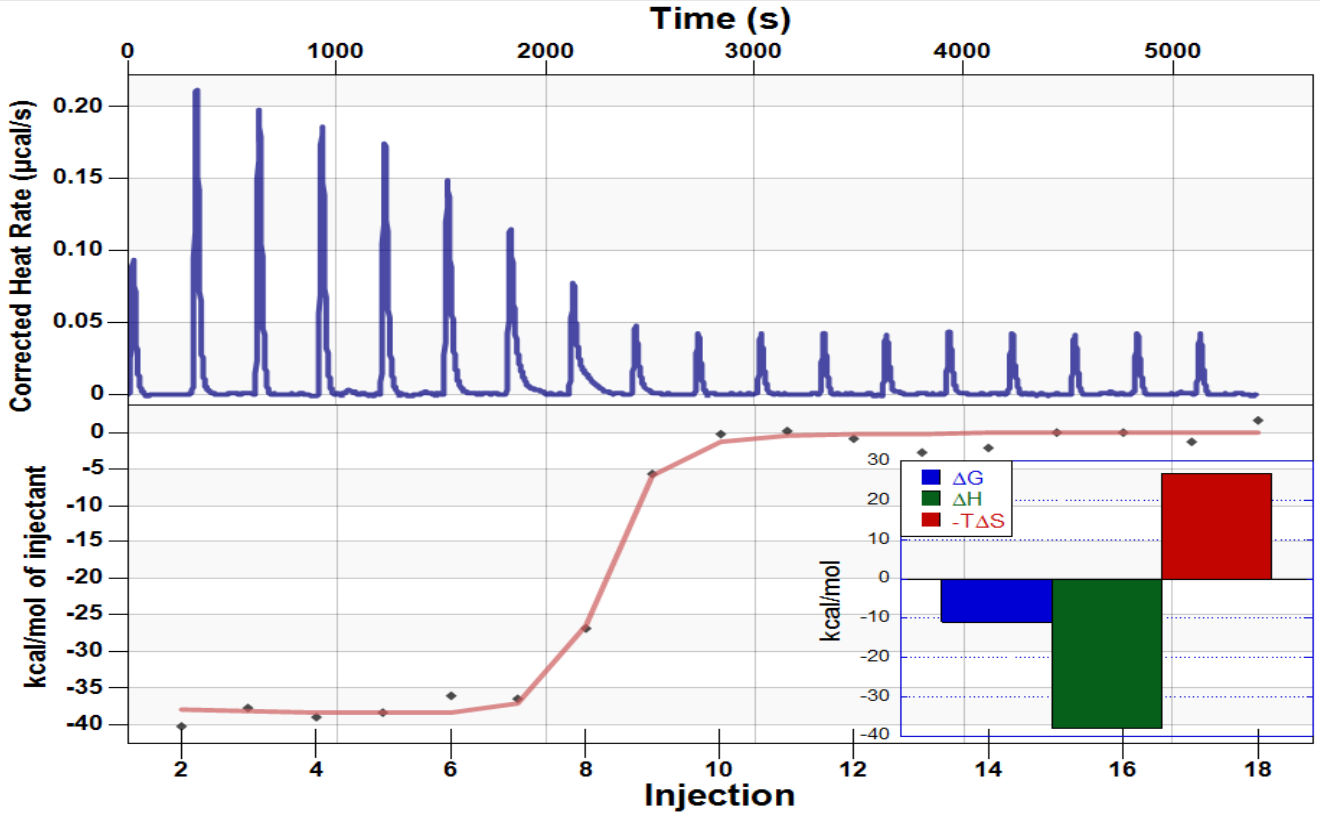
ΔSASA can be calculated from knowledge of ΔC_p (heat capacity change) and ΔH_{60} (enthalpy change at 60°C) by solving the following simultaneous equations for $\Delta SASA_{polar}$ and $\Delta SASA_{apolar}$:

$$\Delta C_p = a[\Delta SASA_{polar}] + b[\Delta SASA_{apolar}]$$
$$\Delta H_{60} = c[\Delta SASA_{polar}] + d[\Delta SASA_{apolar}]$$

where a, b, c and d are semi-empirically determined co-efficients

But Beware!
Anomalously large changes in ΔC_p can also result from linked equilibria such as protonation and entrapment of water molecules within interfacial cavities upon macromolecular associations

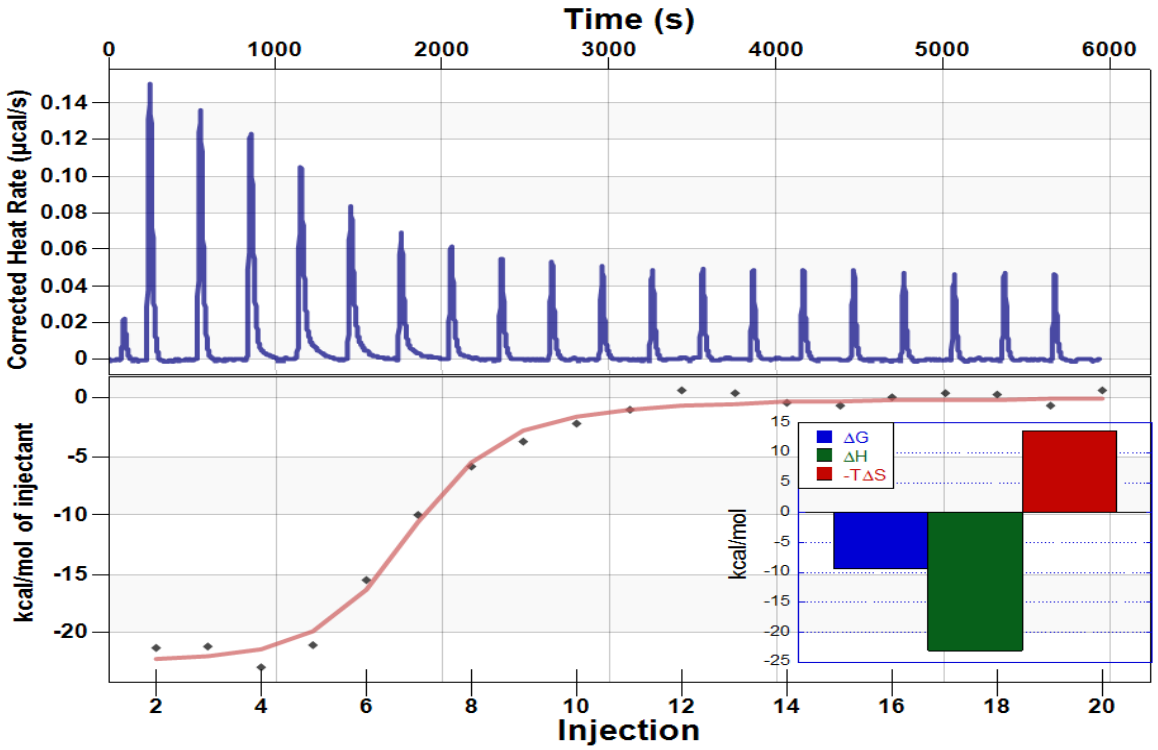
Technique 7: Screening Protein-Protein Inhibitors



- Titration of sCD4 into gp120 using Nano ITC LV
- K_a, = 1.2 × 10^8 M^{-1} ; K_d = 1/K_a of 8.3 nM
- Binding enthalpy, ΔH, is -38.0 kcal/mol
- Entropy contribution to the binding Gibbs energy = 27.0 kcal/mol (1 cal = 4.184 joules)

TA Application Note: Freire, E., et. al. 2012

Technique 7: Screening Protein-Protein Inhibitors



$A = K_{app}/K_a$

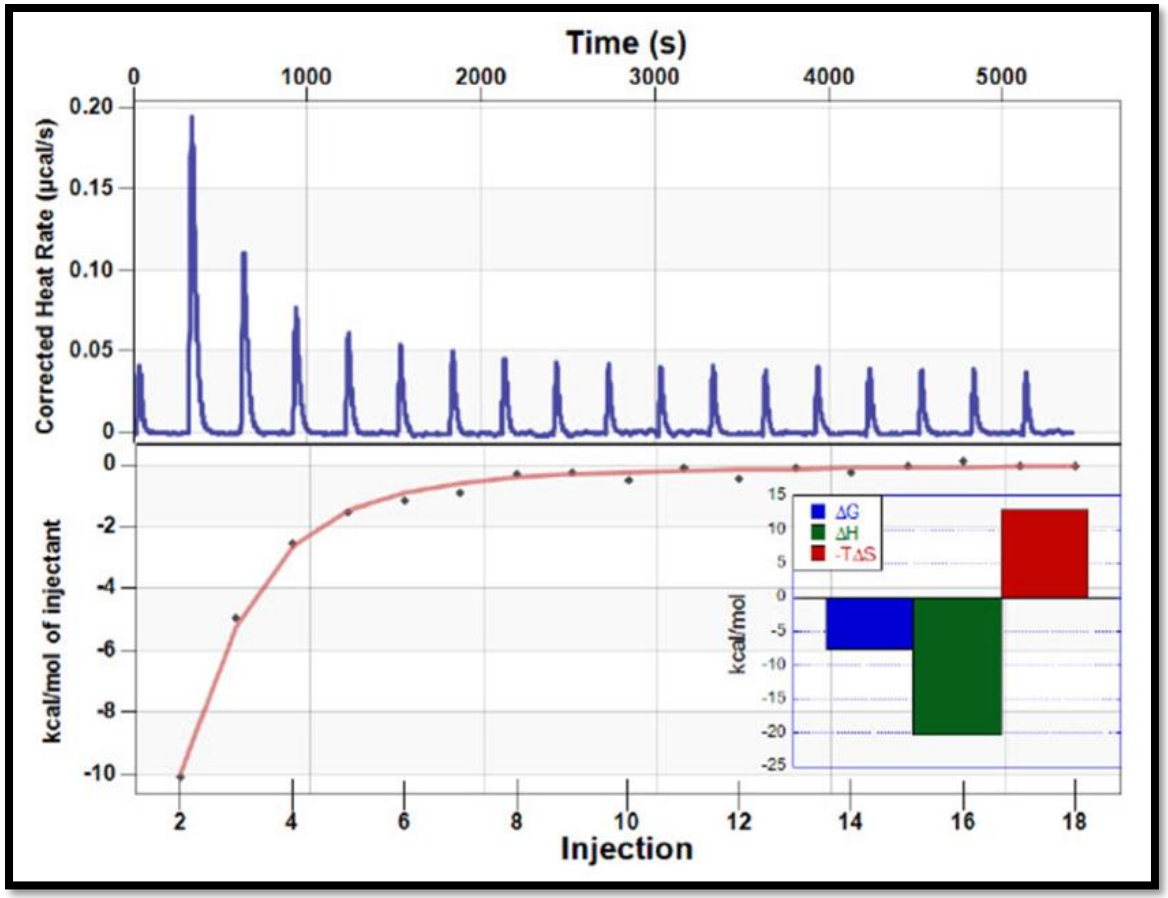
$A = 0.064$

- Titration of sCD4 into gp120 + 200µM of NBD-556, (MW = 337.84); low affinity sCD4/gp120 inhibitor
- K_{app}, = 7.7 × 10^6 M^{-1} ; $K_{d,app} = 1/K_{app}$ of 130 nM
- Apparent binding enthalpy = -23.0 kcal/mol
- Apparent entropy contribution to binding is 13.6 kcal/mol

Technique 7: Screening Protein-Protein Inhibitors

- The magnitude of the decrease can be expressed in terms of the ratio $A = K_{app}/K_a$.
- If A = 1, then the compound has no effect on the protein/protein interaction.
- If A<1, then the compound has an inhibitory effect.
- If all the compounds are screened at the same concentration, then the parameter A suffices to rank them in terms of their inhibitory potency.
- If a compound is found that exhibits an A value greater than one. This compound actually increases the binding affinity acting as an agonist of the protein/protein interaction.

Technique 7: Screening Protein-Protein Inhibitors



- Titration of 300 µM NBD-556 into 5 uM gp120
- K_I, = 3.3 × 10^5 M^{-1} ; $K_{d,I}$ = 1/K_I of 3.0 µM
- Binding enthalpy = -20.4 kcal/mol ;
- Entropy contribution to binding = 12.9 kcal/mol

$$\beta = \frac{A(1+K_I[I])-1}{K_I[I]}$$

β value = 0.05

Technique 7: Screening Protein-Protein Inhibitors

- If β = 0, the inhibitor is absolutely competitive, i.e. either the inhibitor or the protein is bound but not both
-
- If β = 1 the compound does not affect the binding affinity of the protein.

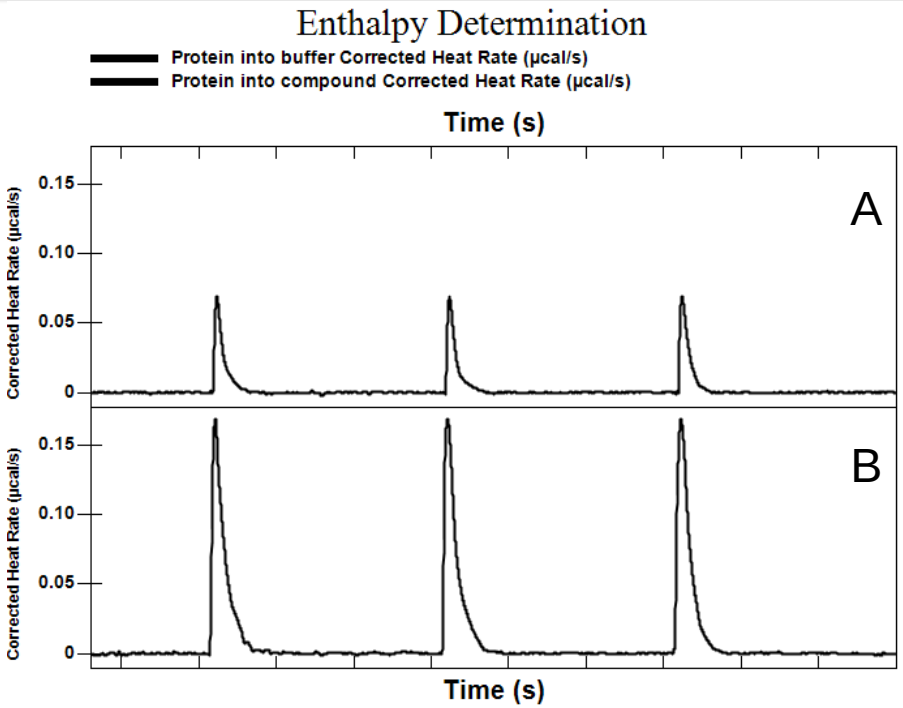
- For small molecular weight compounds β can assume a value between 0 and 1. The presence of the small molecule can be thought off as a mutation that lowers the affinity but not abolishes the binding of the protein for its partner.

- The parameter β can be calculated from the ITC data

Technique 7: Screening Protein-Protein Inhibitors

- NBD-556 is characterized by ITC as a moderately competitive inhibitor
- The optimization of protein/protein inhibitors requires maximization of the binding affinity and modulation of the degree of competitiveness, β. (0.05)
- This guides the development of more or less competitive inhibitors according to the specific design needs.
- Due to the large size of the protein/protein binding footprint when compared to the size of a ~500 MW molecule, inhibitor optimization also requires tracking of the degree of competitiveness since binding affinity alone does not reflect inhibitor potency.
- ITC has unique ability to guide the optimization of protein/protein inhibitors

Technique 8: Enthalpy Screen



Data courtesy of E. Freire & P. Ross: Johns Hopkins University

With Nano-ITC LV:

(A) protein into buffer (Q_B)

(B) protein into compound (Q_P)

Injections: 2.02 µL into 170 µL

$$\Delta H = \frac{Q_P - Q_B}{mols_P}$$

Injection #	Q_P (µcal)	Q_B (µcal)	ΔH (Kcal/mol)	$\frac{Q_P - Q_B}{Q_P}$
1	-15.85	-4.55	-2.33	71%
2	-15.92	-4.49	-2.36	72%
3	-15.93	-4.71	-2.31	70%

The data has been fitted, now what?

Consider what contributes to enthalpy and entropy.

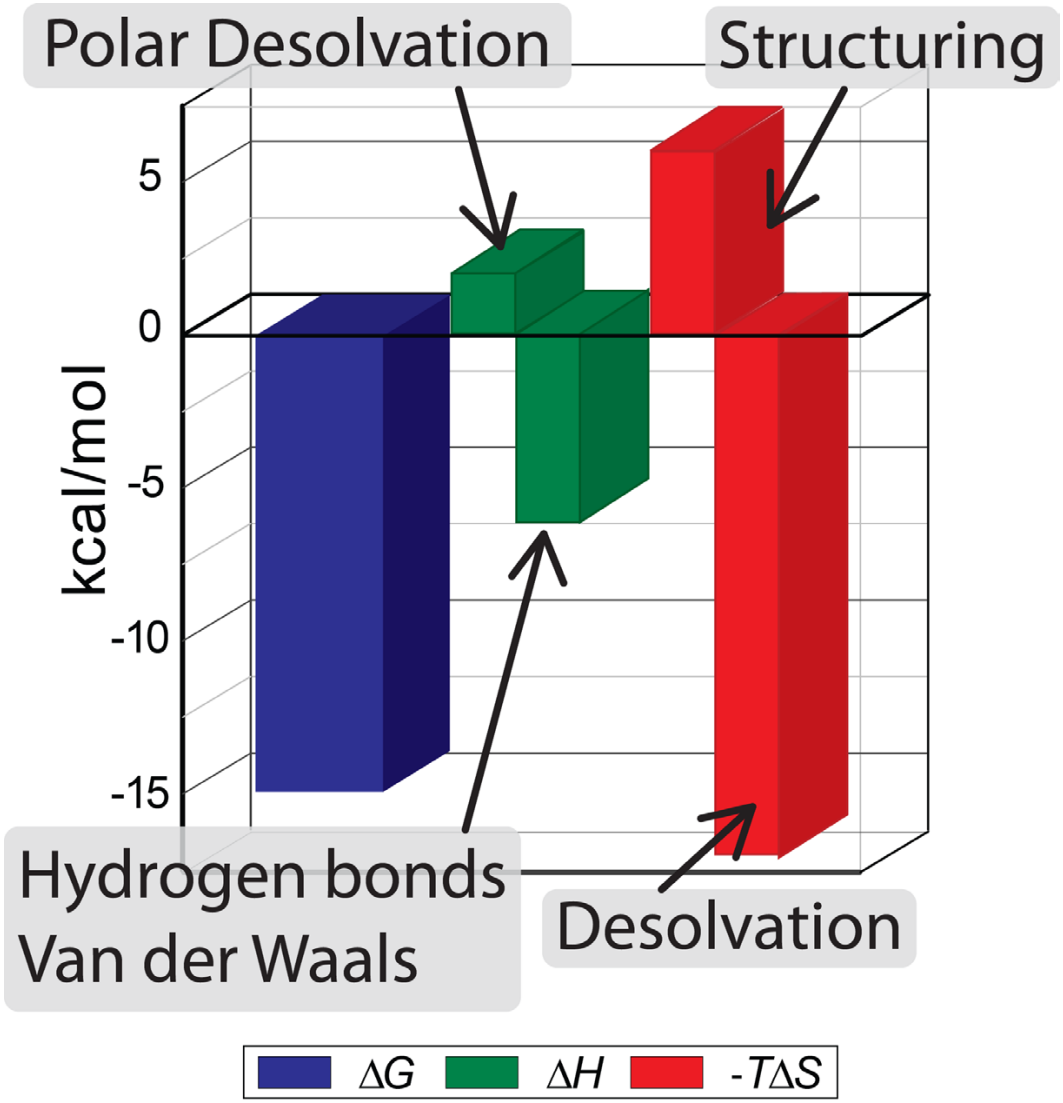
Entropy

- Hydrophobic interactions
- Solvation entropy due to release of water (favorable)

Enthalpy

- Directly associated to number and strength of H-bonds broken or formed
- Choice of solvent important

Different Contributions to Binding

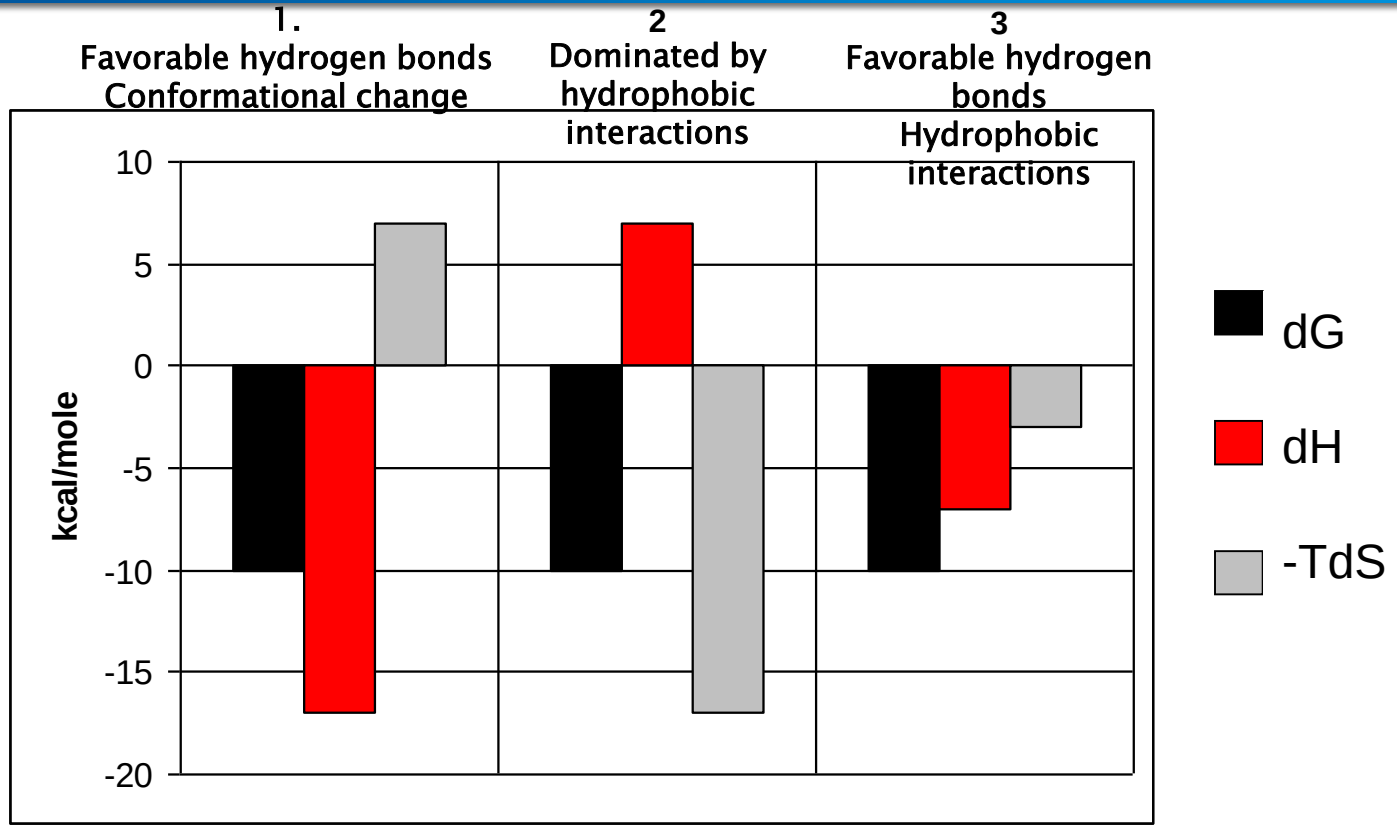


Remember

$\Delta G = -RT \ln K_a$

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

Assigning "Why"



Entropy

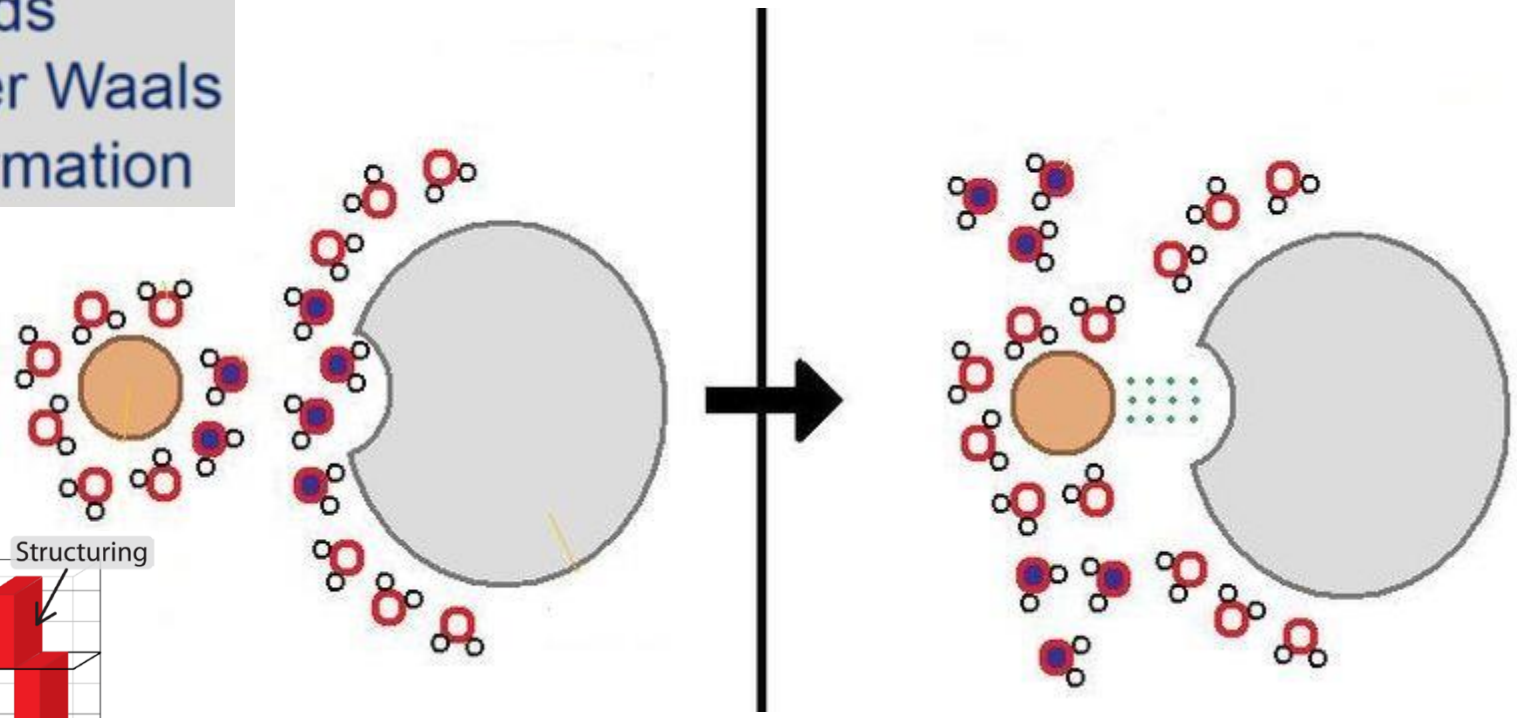
- Hydrophobic interactions
- Solvation entropy due to release of water (favorable)

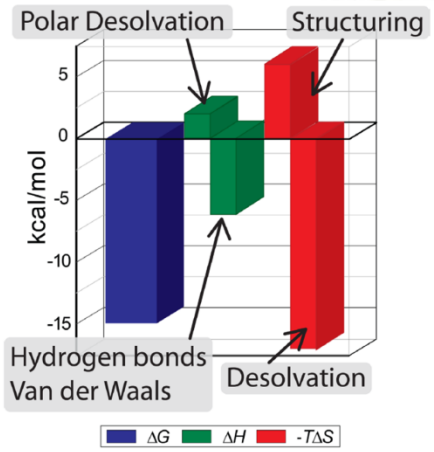
Enthalpy

- Directly associated to number and strength of H-bonds broken or formed
- Choice of solvent important

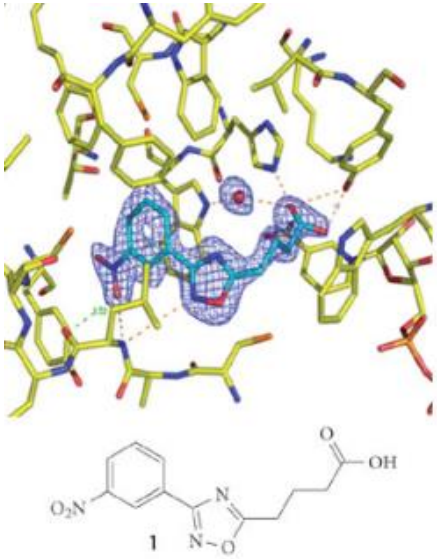
Enthalpy and Entropy "describe binding"

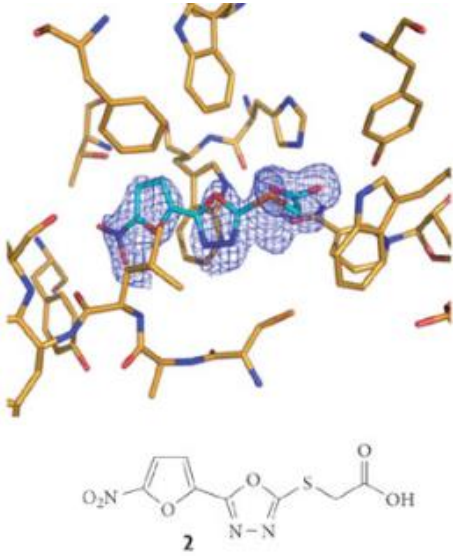
- Desolvation
- H-Bonds
- Van der Waals
- Conformation

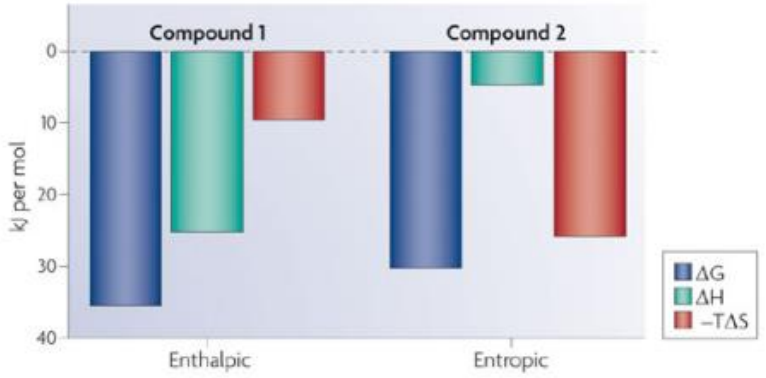




Example of how Enthalpy and Entropy "describe binding"







Ladbury, Klebe, Freire. *Nature Reviews*. 2010. Vol 9, pp 23-27

$\Delta G/K_a$ is similar.

1. Δ H is 5x greater for compound 1.

Why?

Compound 1: H-bonding of water in pocket.

Compound 2: ΔH penalty because of polar desolvation

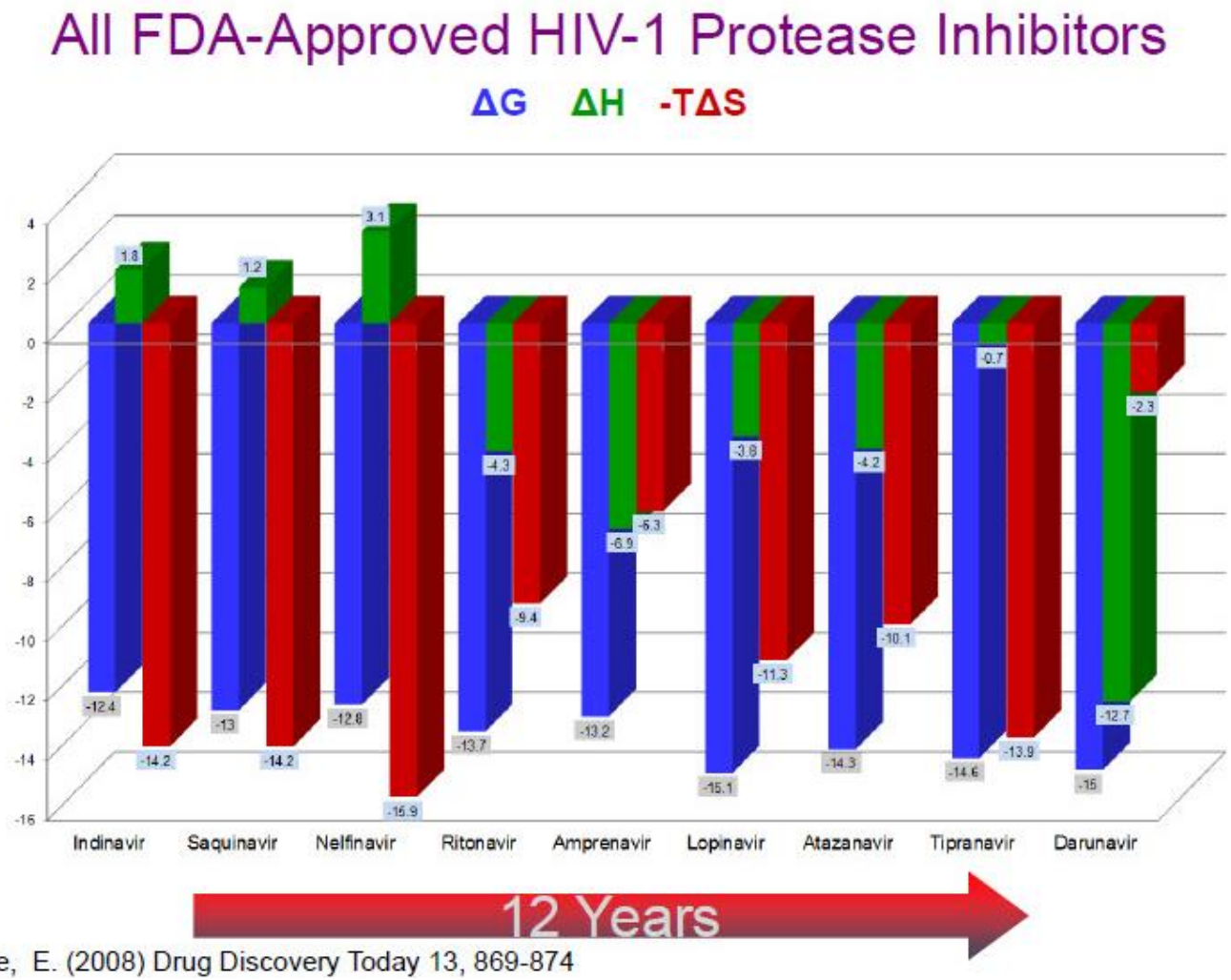
2. Entropy is more favored for compound 2.

Why?

Compound 1 requires structuring

Compound 2 more desolvation

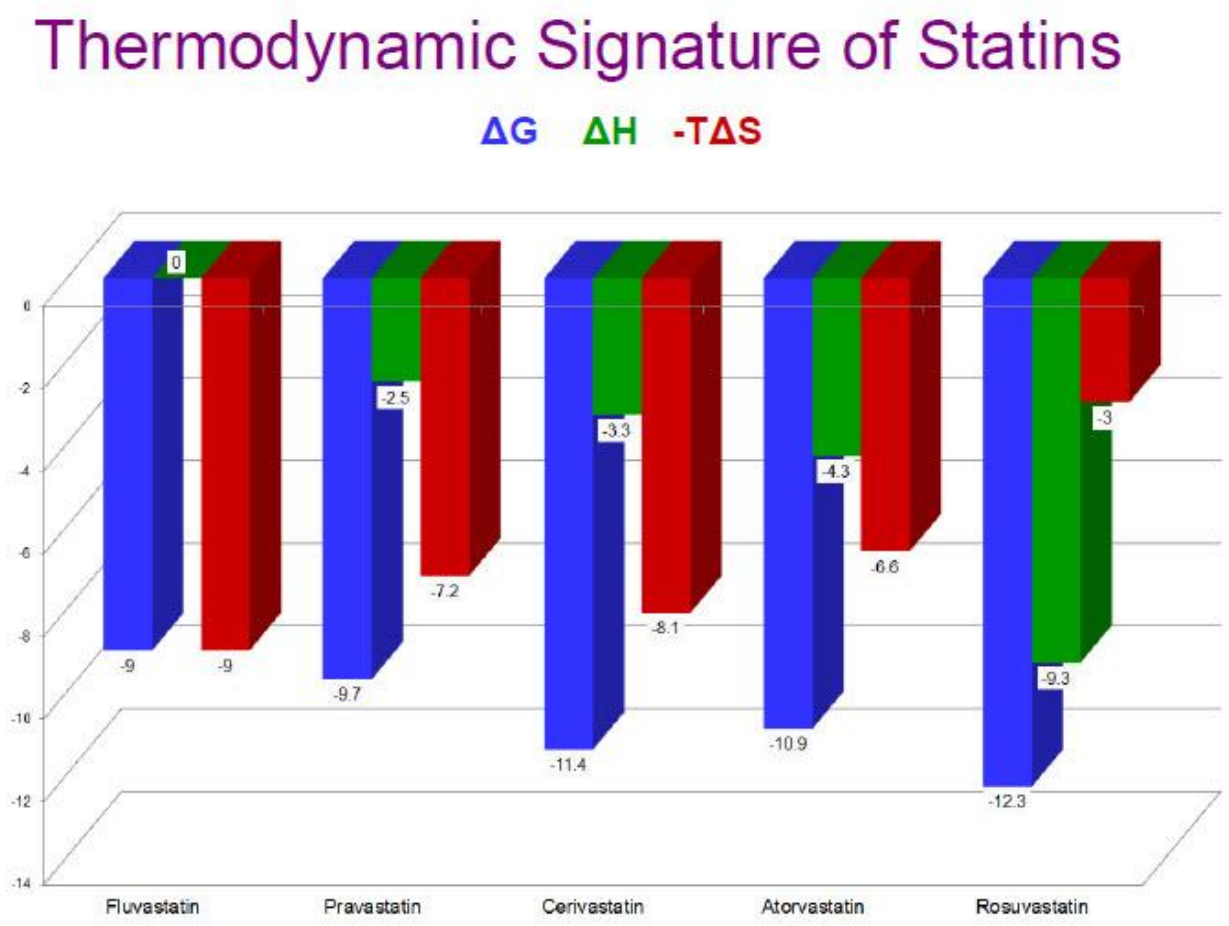
ITC: Biopharmaceutical Characterization



ΔH = Specificity

Freire, E. (2008) Drug Discovery Today 13, 869-874

ITC: Biopharmaceutical Characterization



Over time statin drugs have also had less side-effects – correlation?

Freire, E. (2008) Drug Discovery Today 13, 869-874

Questions?

Obtaining meaningful ITC binding data: NanoAnalyze Experiment Design

NanoAnalyze Experiment Design - Demonstration

Experiment Setup

Obtaining Meaningful ITC Binding Results: Choosing Correct Concentrations

- Choosing correct ligand and receptor concentrations requires an estimate of stoichiometry and K_a, often from spectroscopic measurements. ITC is then used to determine K_a accurately
  - Weak binding (low K_a) – may be limited by concentration
    - may use multiple syringes and combine results
  - Strong binding (high K_a)
    - minimize concentration or injection volume
    - try competitive binding (displacement) experiment

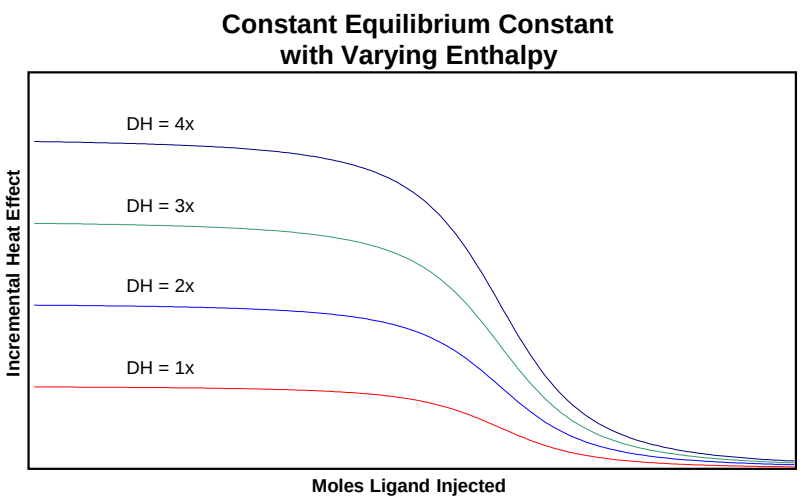
- Use Experiment Design module to alter K_a, binding model, stoichiometry and concentrations, and see the effect on the binding curve. Requires 'best guess' inputs of stoichiometry, K_a and binding model

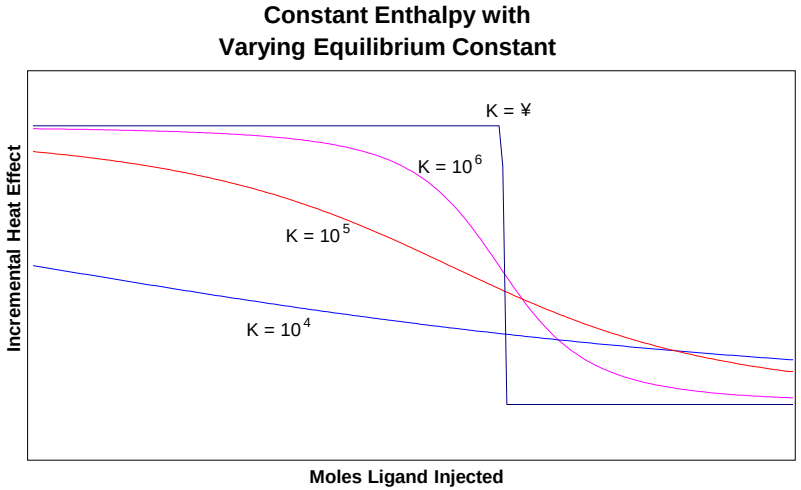
- Unsure about the parameters – NanoAnalyze Experiment Design

Obtaining Meaningful ITC Binding Results

$$A + B \underset{\Delta H}{\overset{K}{\rightleftharpoons}} AB$$

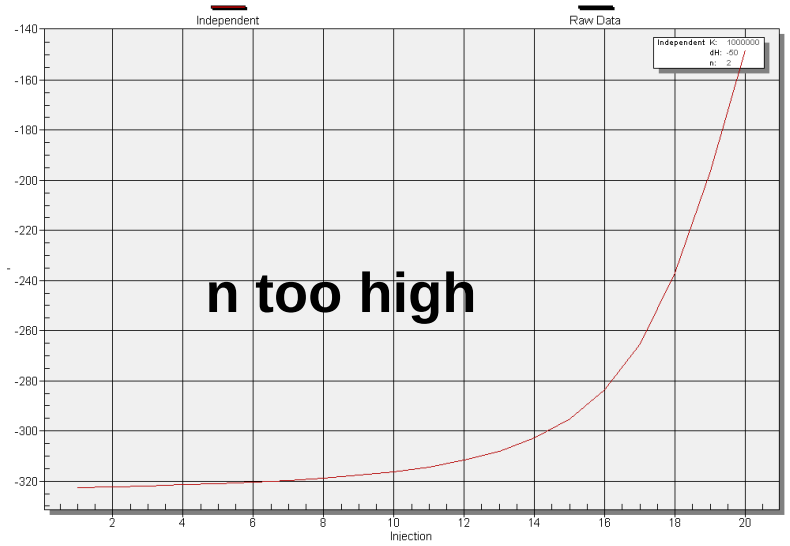
$$K = \frac{[AB]}{[A][B]}$$

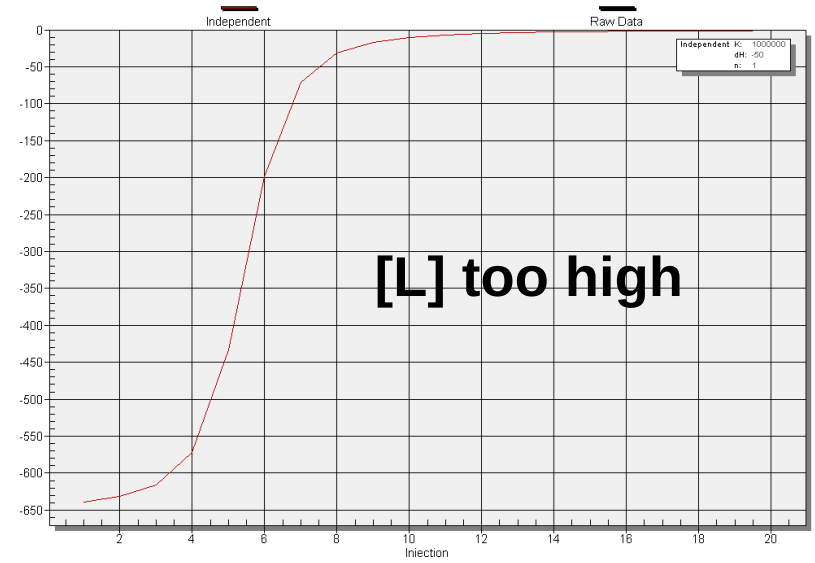


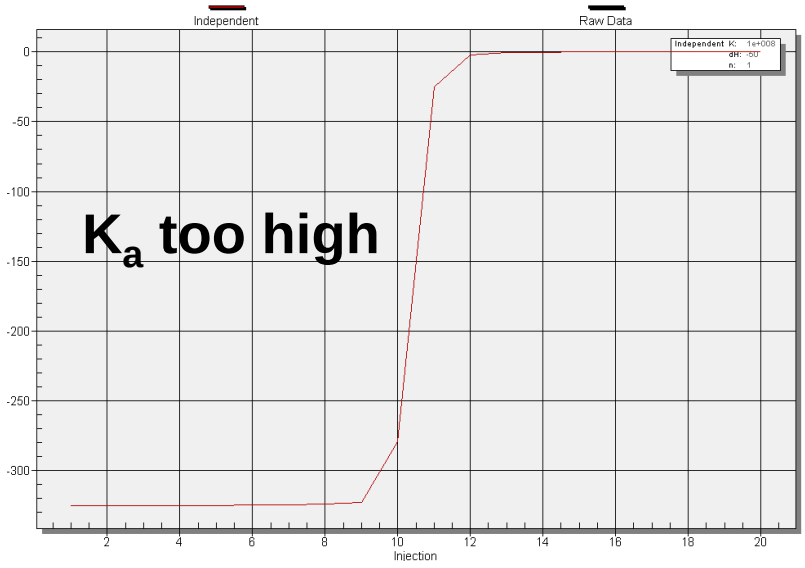


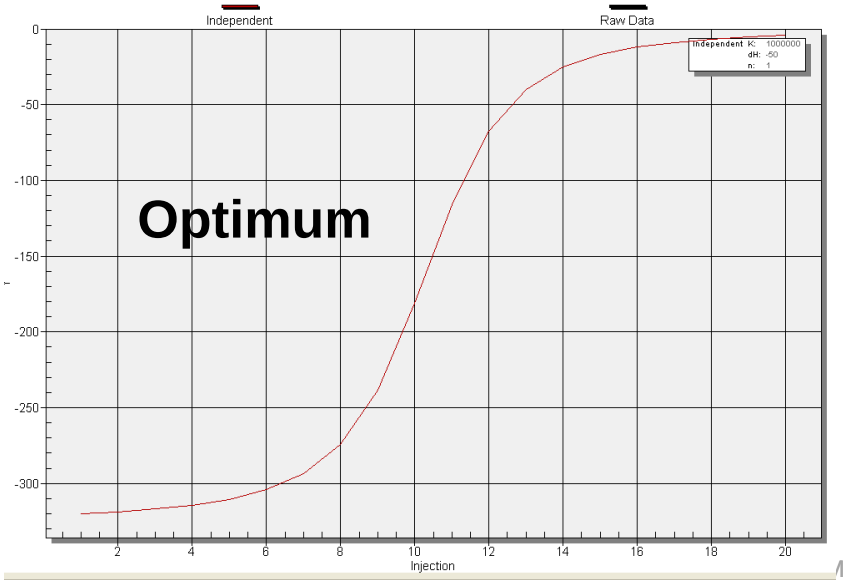
- Generally want to obtain stoichiometry (n), enthalpy (ΔH) and binding constant (K_a) from one experiment
- Enthalpy is directly measured. Receptor should be saturated with ligand at end of titration
- To obtain K_a: $10 < K_f C_R < 1000$
- C_R is typically 10 – 100 mM, K_a is typically 10^3 to 10^9 M^{-1}

Obtaining Meaningful ITC Binding Data: NanoAnalyze Experiment Design









Obtaining Meaningful ITC data: Sample Preparation

- All reactions produce heat. Ensure only the desired reaction is measured.
  - pH, ionic strength, choice of buffer and temperature
- Diluting a compound produces or absorbs heat ('heat of dilution').
  - To minimize heat of dilution of buffer 'contaminants' in protein and ligand solution: 1) dialyze protein and ligand 2) use the used dialysis buffer to dissolve the ligand 3) perform 'blank' experiment (titrate ligand into buffer), then subtract blank from 'real experiment' data. Caution: analyze blank data for indications of ligand-buffer interactions.
  - Dialyze samples to minimize heat of dilution.
- Determine concentration and purity
  - Multiple techniques (i.e. Spectrophotometricly and SDS-PAGE)
- Degas samples if necessary

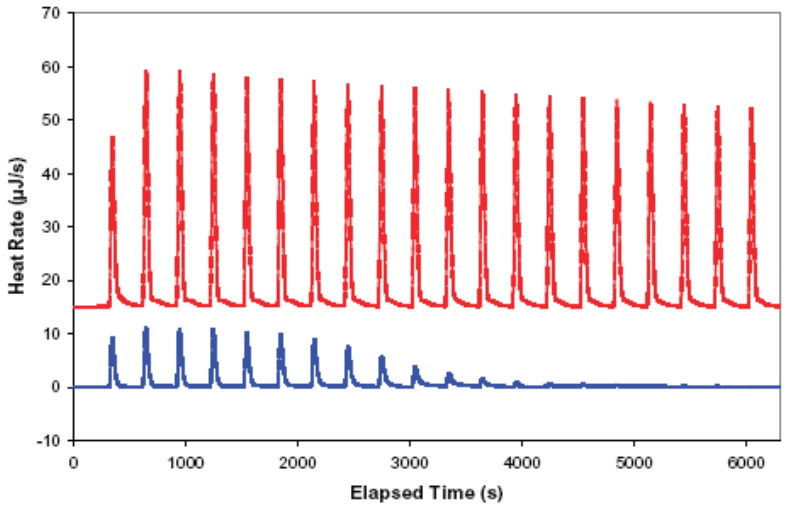
Obtaining meaningful ITC data: Experiment Optimization

- Small heat of binding? Change temperature, pH and/or buffer
- Temperature: ΔH is temperature and system dependent, varies with ΔC_p. Conduct experiment at relevant temperature where binding has measurable ΔH.
- Different buffers have different enthaplies of ionization, affect ΔH of binding. If low enthalpies observed, use buffer with high ionization enthalpy
- Low enthalpy may indicate that a non-optimal pH is being used.

ITC is like any other experimental technique: Requires Optimization

Obtaining Meaningful ITC Data: Organic Solvents/Additives

- Reducing agents
  - Use TCEP instead of DTT or β-mercaptoethanol
- Solubility problems
- Non-aqueous solvents have different viscosities and heat capacities.
- Match [organic] in reference and sample cell?
- Any mismatch in [organic] can result in
  heat of dilution masking
  heat of binding
- Example: 5% DMSO
  decreases affinity of RNase A.



Obtaining Meaningful ITC Data: Unexpected Stoichiometry

- Unexpectedly low stoichiometry could be due to:
  - [receptor] lower than anticipated
  - [ligand] higher than anticipated
  - Receptor contains contaminating proteins
  - Receptor is partially unfolded
  - Multiple binding sites
  - Wrong binding model
  - Insufficient curvature in data: change concentrations
  - Solvent protonation
    - Study different buffers at the same pH

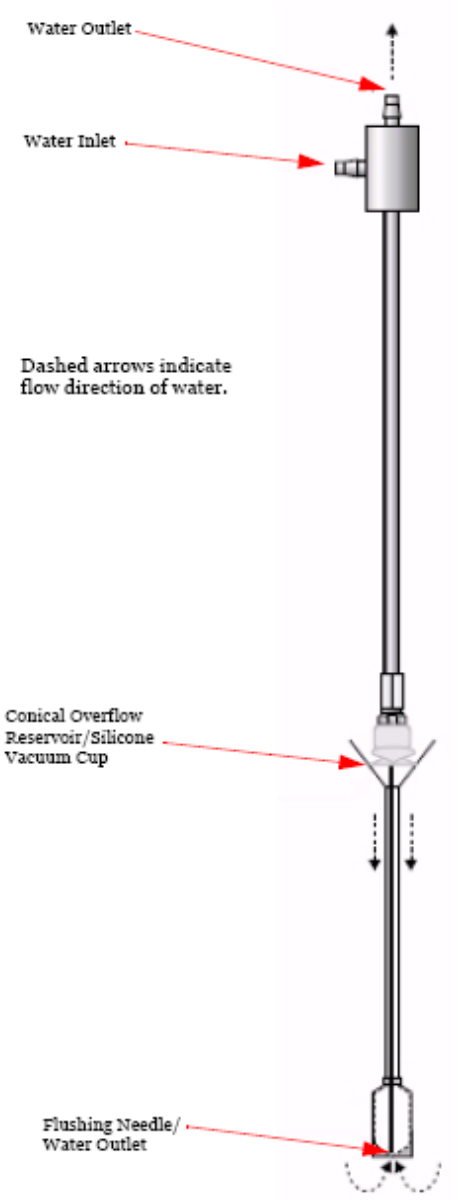
Obtaining Meaningful ITC Data: Obvious, But Often Forgotten

- Ensure syringe is scrupulously clean to eliminate titration of contaminants and ensure reproducible delivery of titrant. No air bubbles
- Clean cell aggressively as required
- To minimize syringe-tip diffusion, divide equilibration into three phases: sample equilibration (no syringe); equilibration with syringe (no stirring), equilibration with stirring
- Ensure baseline is stable before proceeding to the next step
- Stirring speed is a compromise: increased speed increases noise, but too slow stirring causes trailing in binding data.
- Does binding occur within seconds (or a least minutes)?

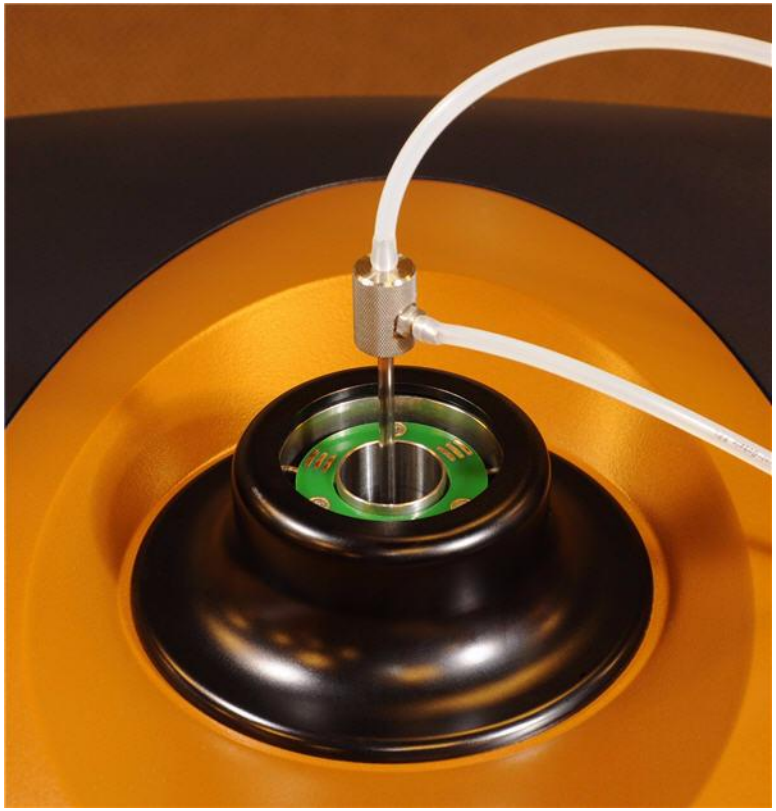
ITC Instruments

Focus on Cleaning

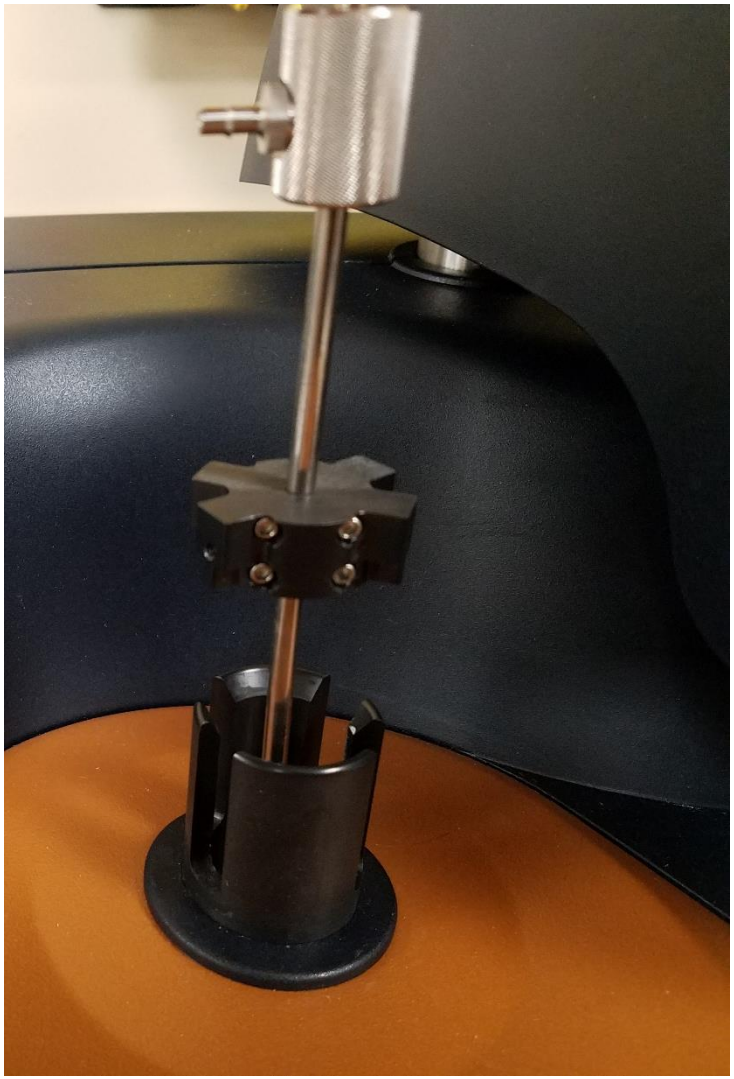
Nano ITC Maintenance



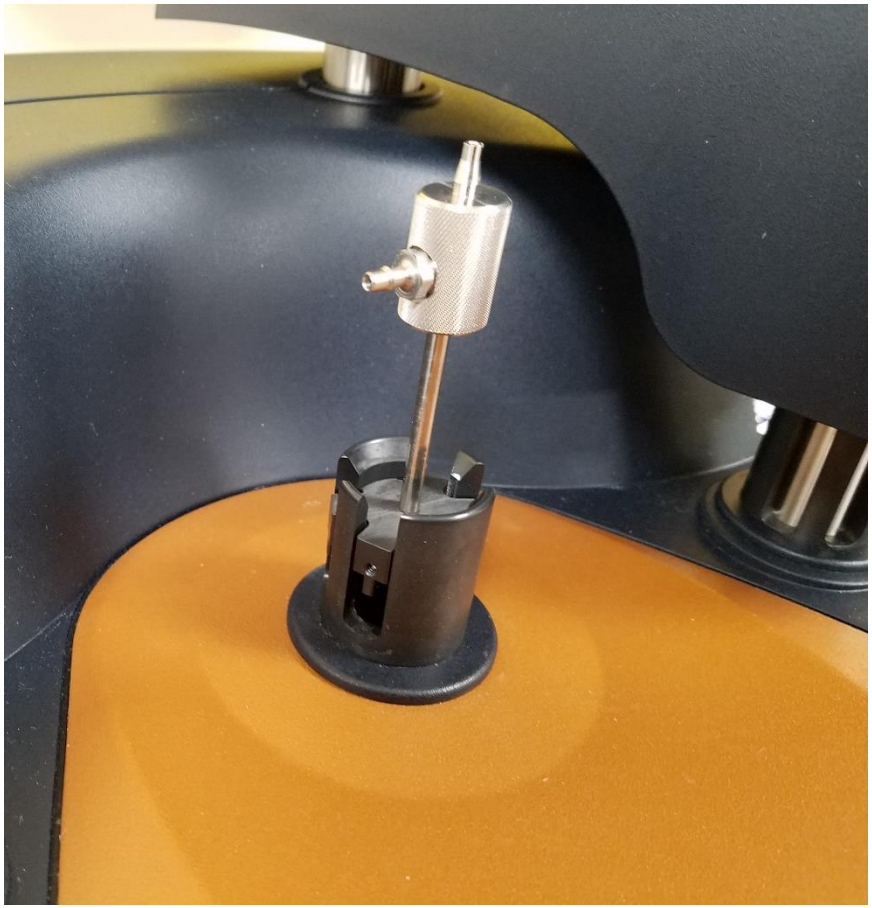
- Easy-to-use cleaning tool for simple and rapid preparation of the ITC cell for another sample
- High-volume flushing of sample cell with Degassing Station accessory



Affinity ITC Maintenance



- Same tool, but with a fixture at the top to hold it steady within the alignment tab.



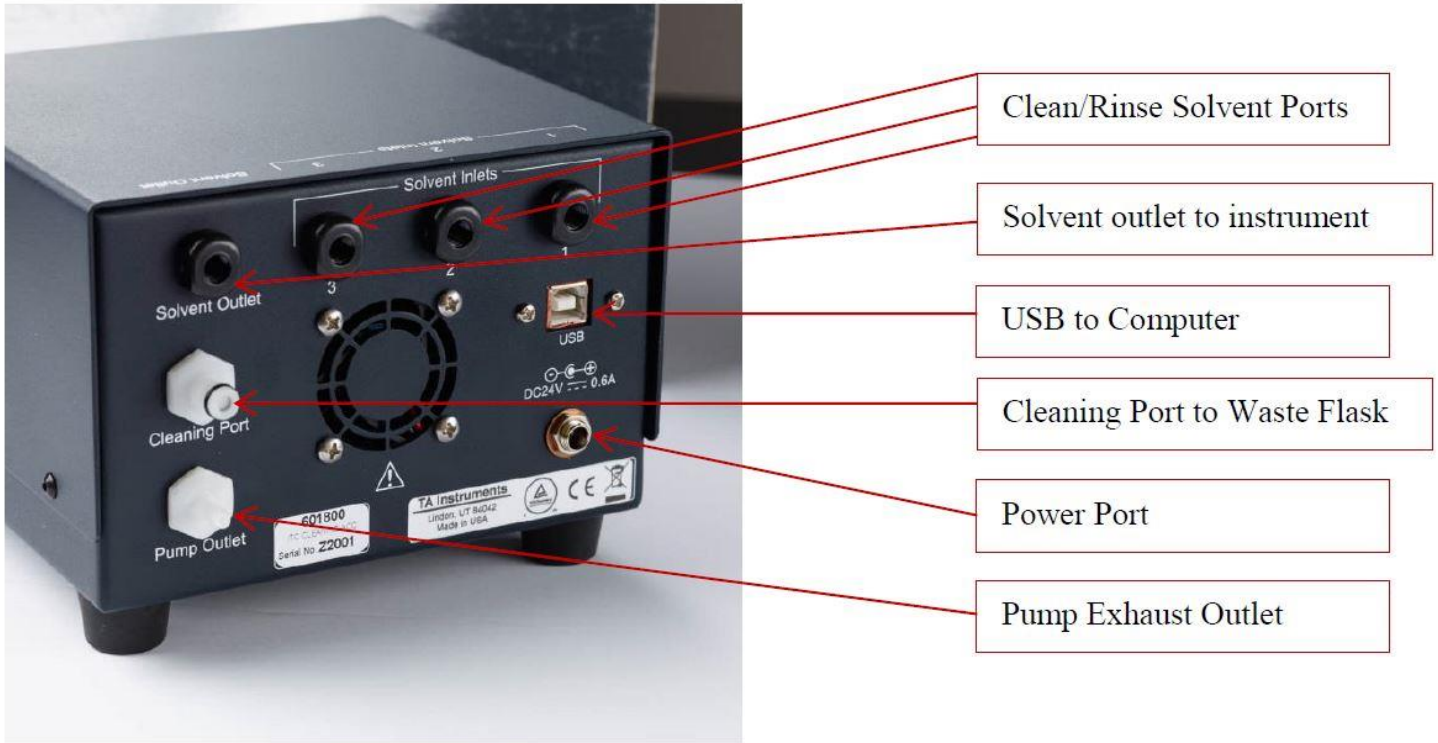
Cleaning Station

- Cleaning station allows you to program up to 3 different cleaning solutions with volumes and several steps. Works with cleaning tool and sidearm flask.

Cleaning Station

- Cleaning station allows you to program up to 3 different cleaning solutions with volumes and several steps. Works with cleaning tool and sidearm flask.

Rear Panel Connections:



Cleaning your ITC cell

- Contrad 70
  - Rinses cleanly
  - Very low concentration in syringe (0.8% and rinse with methanol then water)
  - 0.8%-2% in the cell
- 4N NaOH
- 50% Formic acid
  - Can use up to 4N Nitric acid
- For Lipids, use ethanol or methanol
- Can use Peptidases for proteins
- Use appropriate solvents for your chemistry
  - Try to avoid Toluene, pay attention to miscibility
  - May want to go through Methanol or Acetone to get to hydrophobic solvents

My Cleaning Protocol - Cell

- Start with 5% Contrad-70, 20% Methanol, 1M NaOH
  - Rinse cell with cleaning syringe
- 4N NaOH, heat cell to 65ºC for 30-60 min
  - Cover cell
- Cool cell back to 25ºC, then rinse with 300-500 mL DI water
  - **Rinsing room temp fluids through a hot cell can damage sensors**
- 50% Formic acid, heat cell to 65ºC for 30-60 min
  - Cover cell
- Cool cell back to 25ºC, then rinse with 800 mL DI water
  - **Rinsing room temp fluids through a hot cell can damage sensors**
- Be sure to rinse out your loading syringe well
  - Use a squirt bottle to back fill the syringe and then push out with the plunger. Do this at least 10x

My Cleaning Protocol - syringe

- Try to clean with only buffer and water if possible
- If you need to clean the syringe, use a low concentration of Contrad
  - Typically I use 0.8% Contrad 70
- After washing with the detergent, rinse with some alcohol (Methanol or Ethanol) to break the surface tension of the surfactant.
- Then rinse thoroughly with water
- Run a water into water (or buffer into buffer) experiment to make sure that the syringe is fully rinsed.

My Cleaning Protocol

Run a water into water ITC run to verify that the cell is clean

- Sometimes the first run is not ideal, so run a second without further cleaning.
- If the peaks have a larger heat at first then come down, it may indicate that your syringe is not completely rinsed

You have stripped the ions from the cell with this aggressive cleaning. Make sure that you rinse the cell several times with buffer before loading sample to condition the cell.

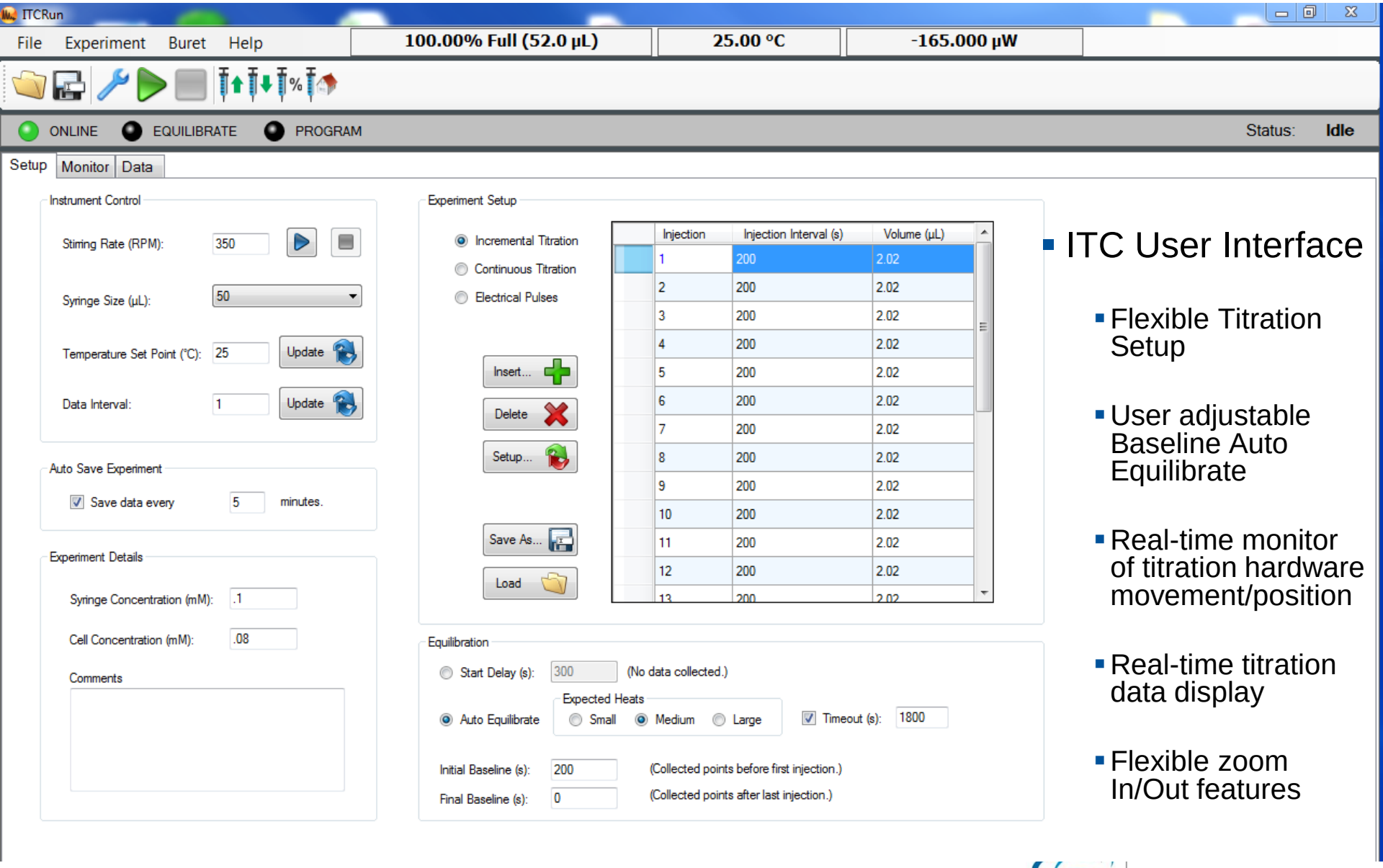
I recommend running this cleaning procedure anytime you are having issues or a suspect baseline. This is probably worth running every 3-6 months as a preventative maintenance procedure as well.

ITC

Data Collection and Analysis Software

TA

ITCRun - ITC Data Collection Software

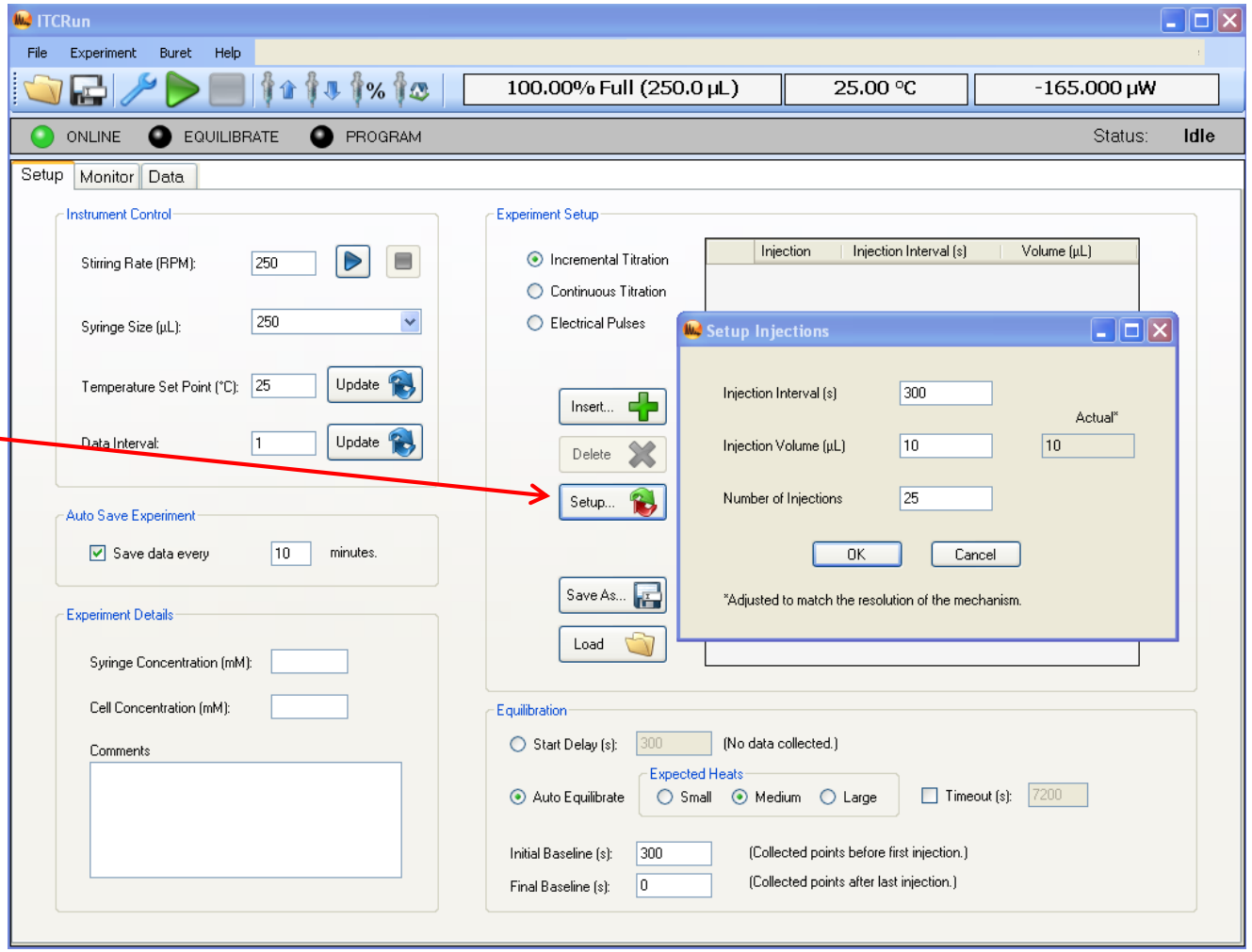


- ITC User Interface
  - Flexible Titration Setup
  - User adjustable Baseline Auto Equilibrate
  - Real-time monitor of titration hardware movement/position
  - Real-time titration data display
  - Flexible zoom In/Out features

ITCRun Experiment Setup

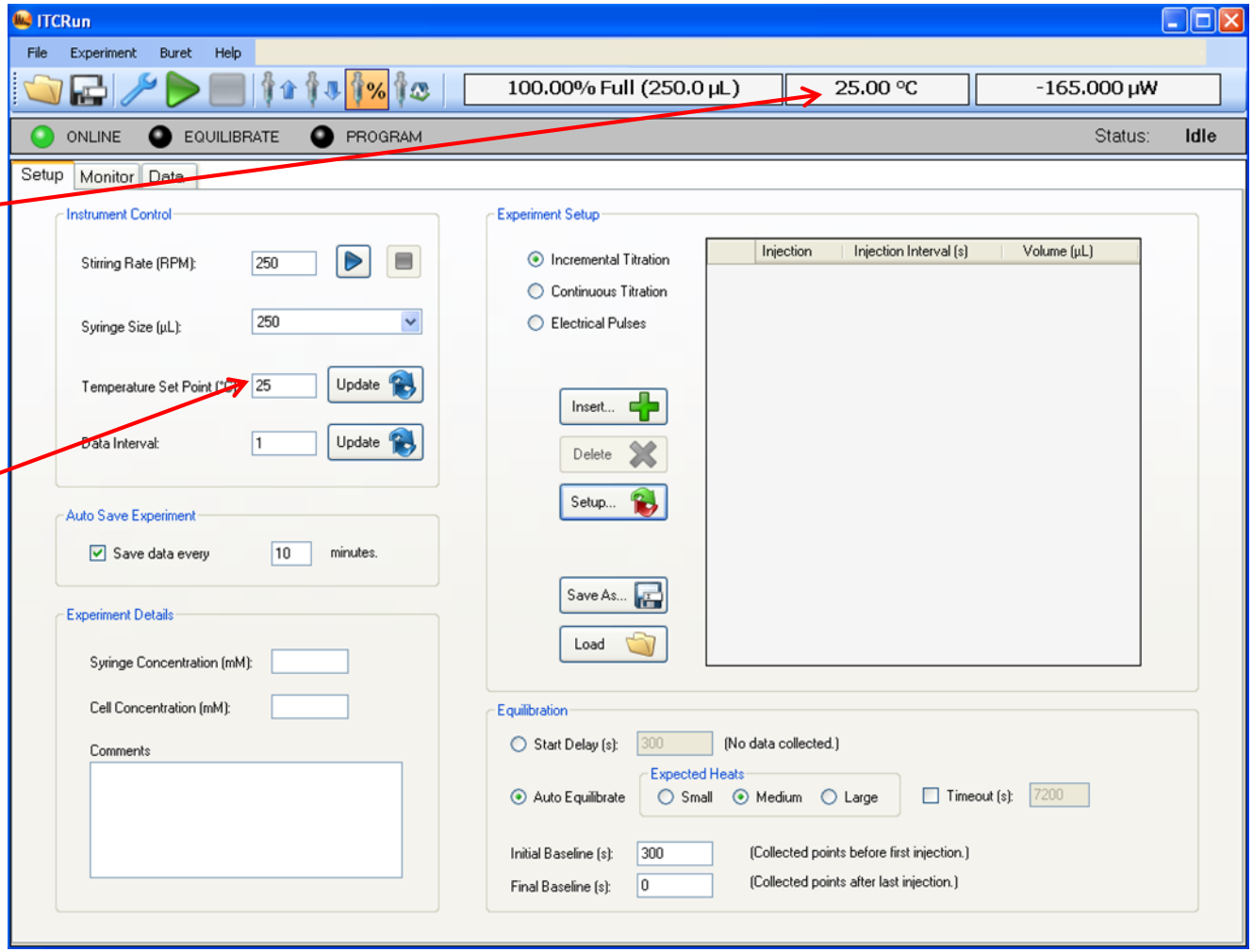
- **Set the injection parameters**

Set method



ITCRun Experiment Setup

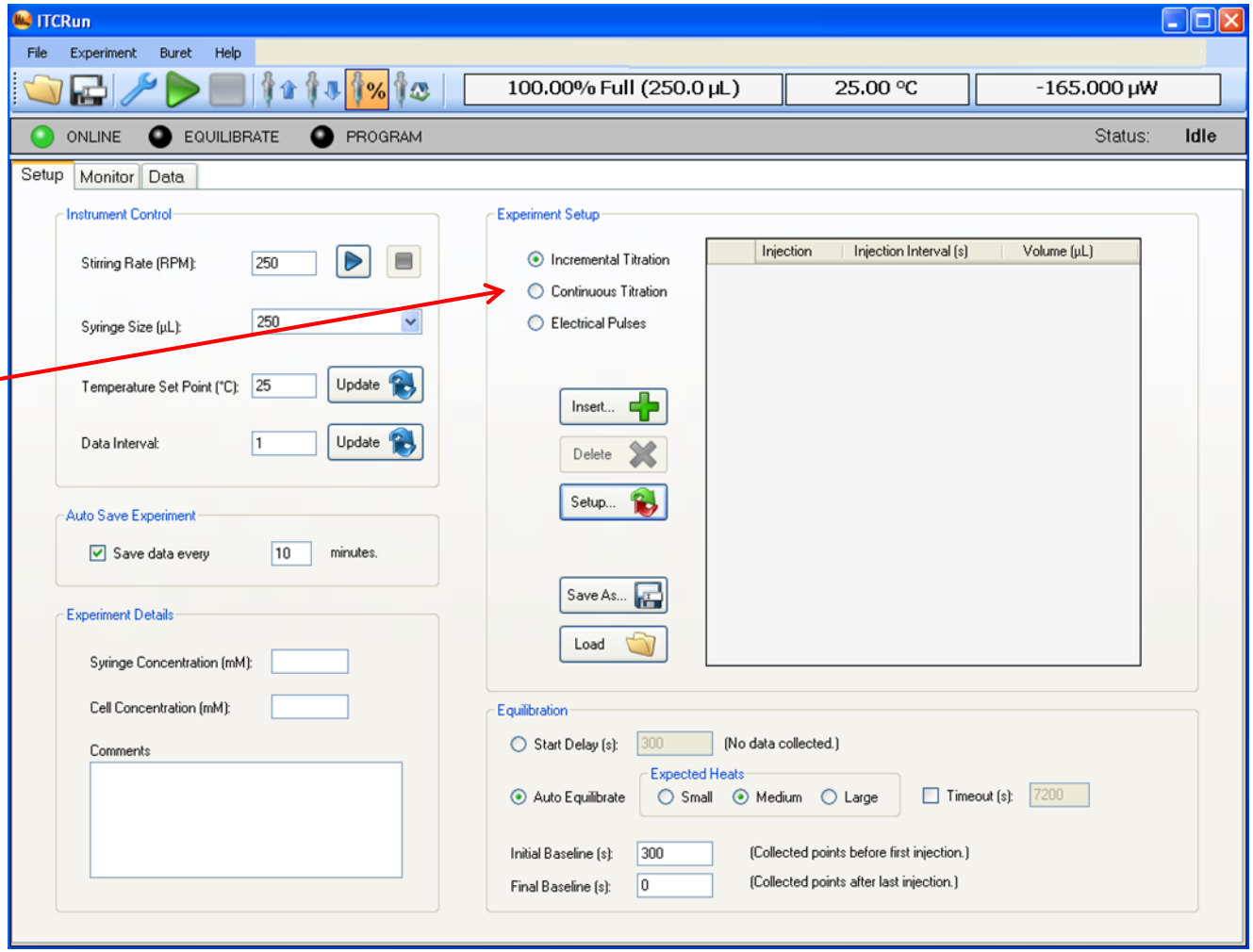
- **Monitor/check temperature**



ITCRun Experiment Setup

- **Select the experiment type**

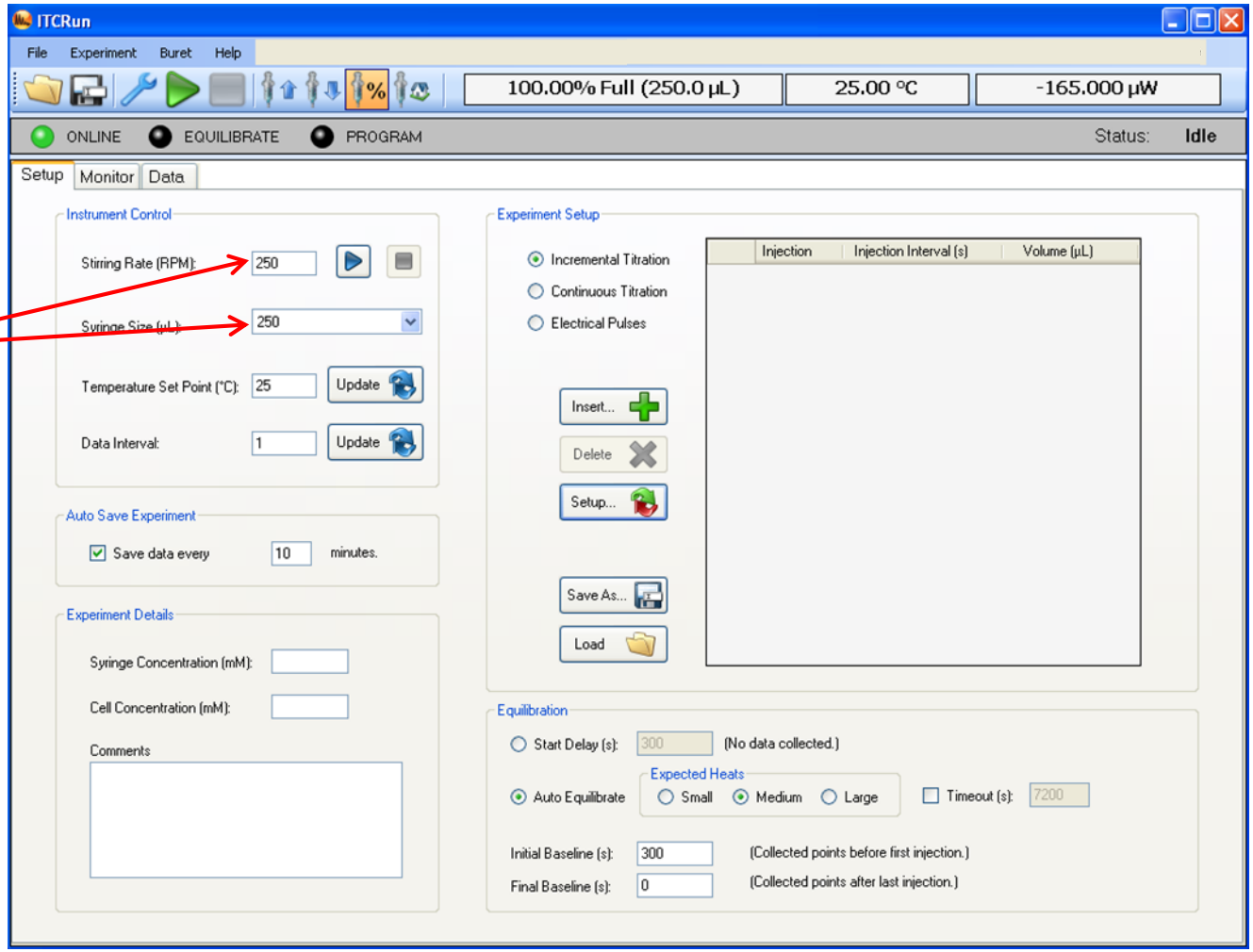
Select Experiment type



ITCRun Experiment Setup

- **Set the syringe size and stirring speed**

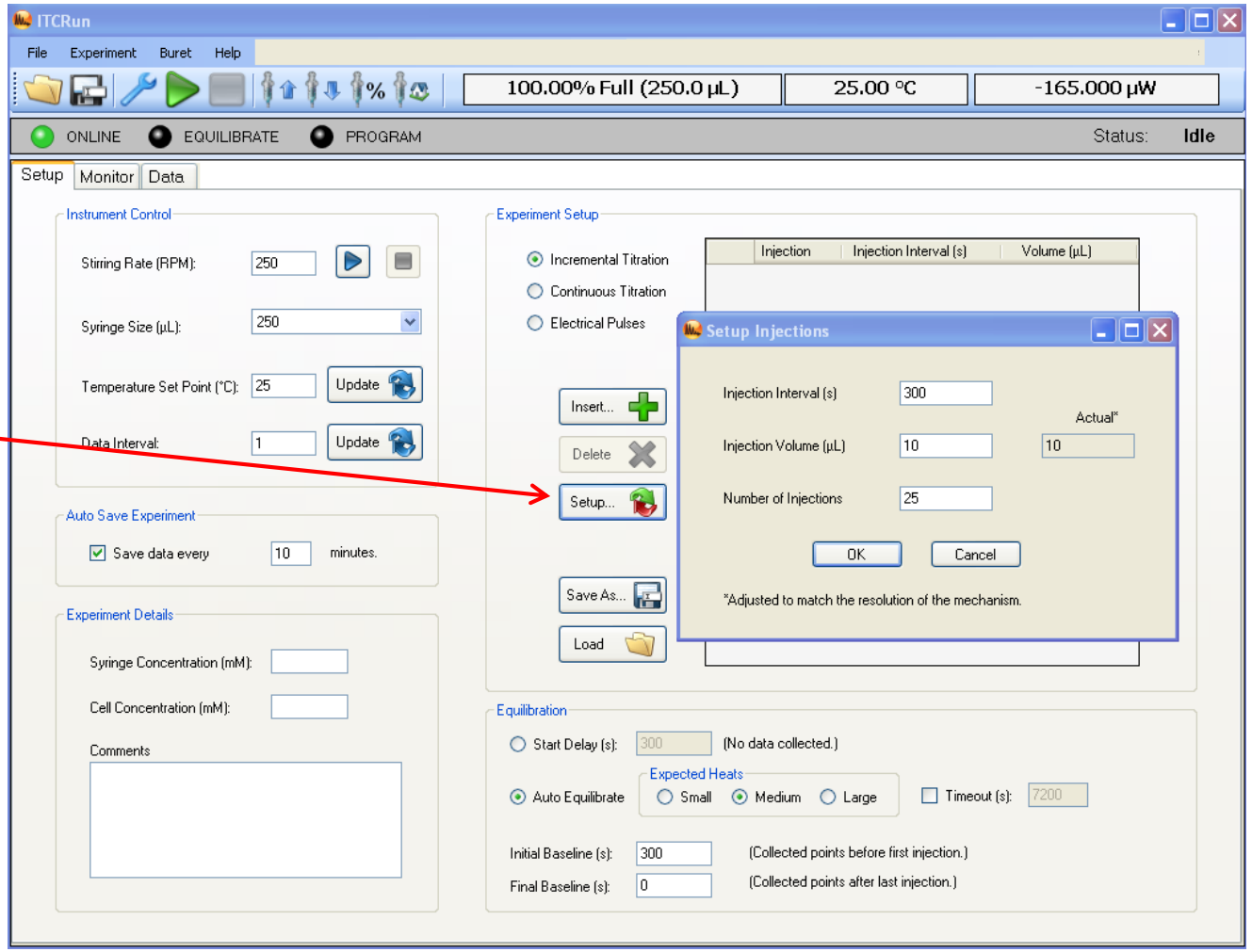
Stir speed and syringe size



ITCRun Experiment Setup

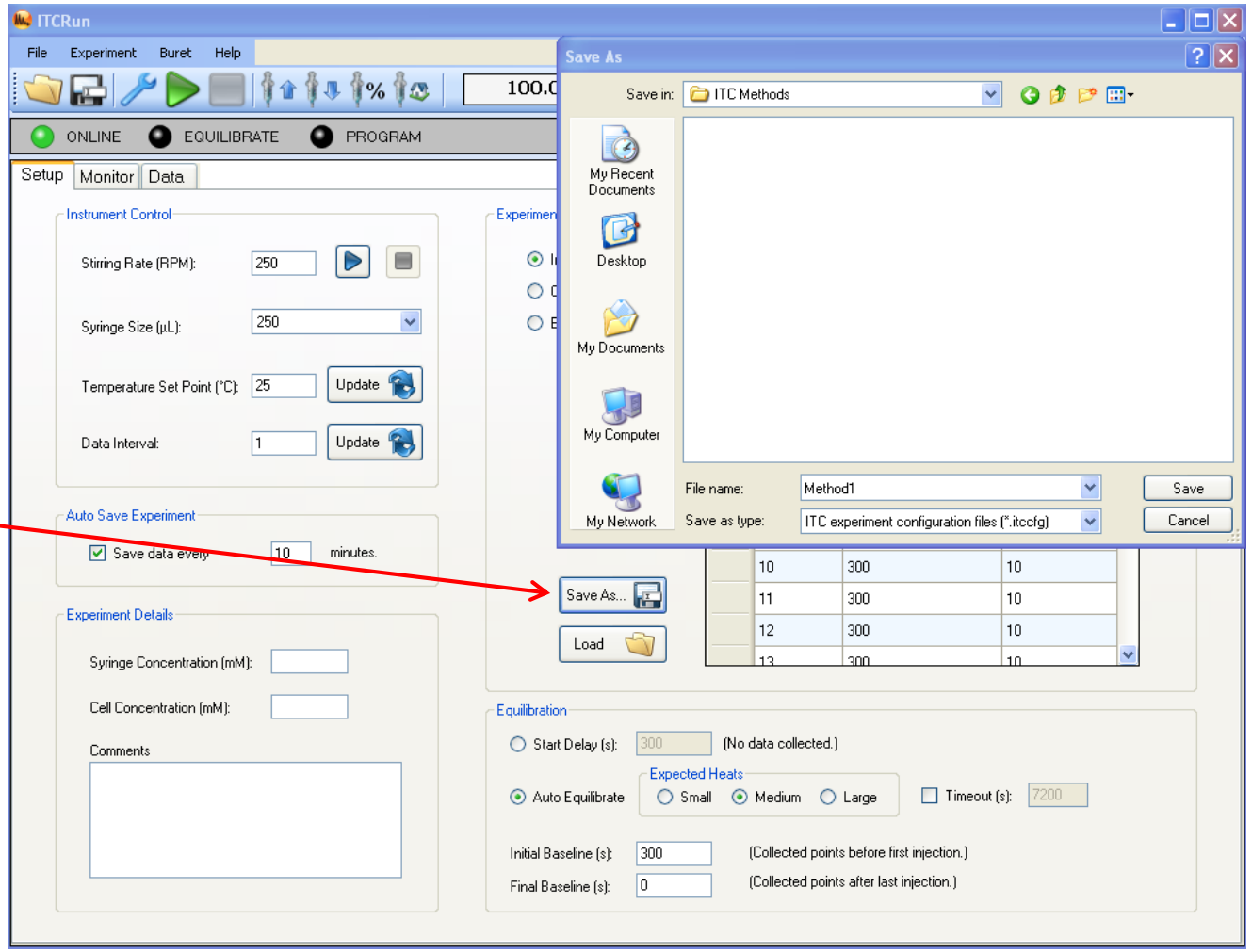
- **Set the injection parameters**

Set method



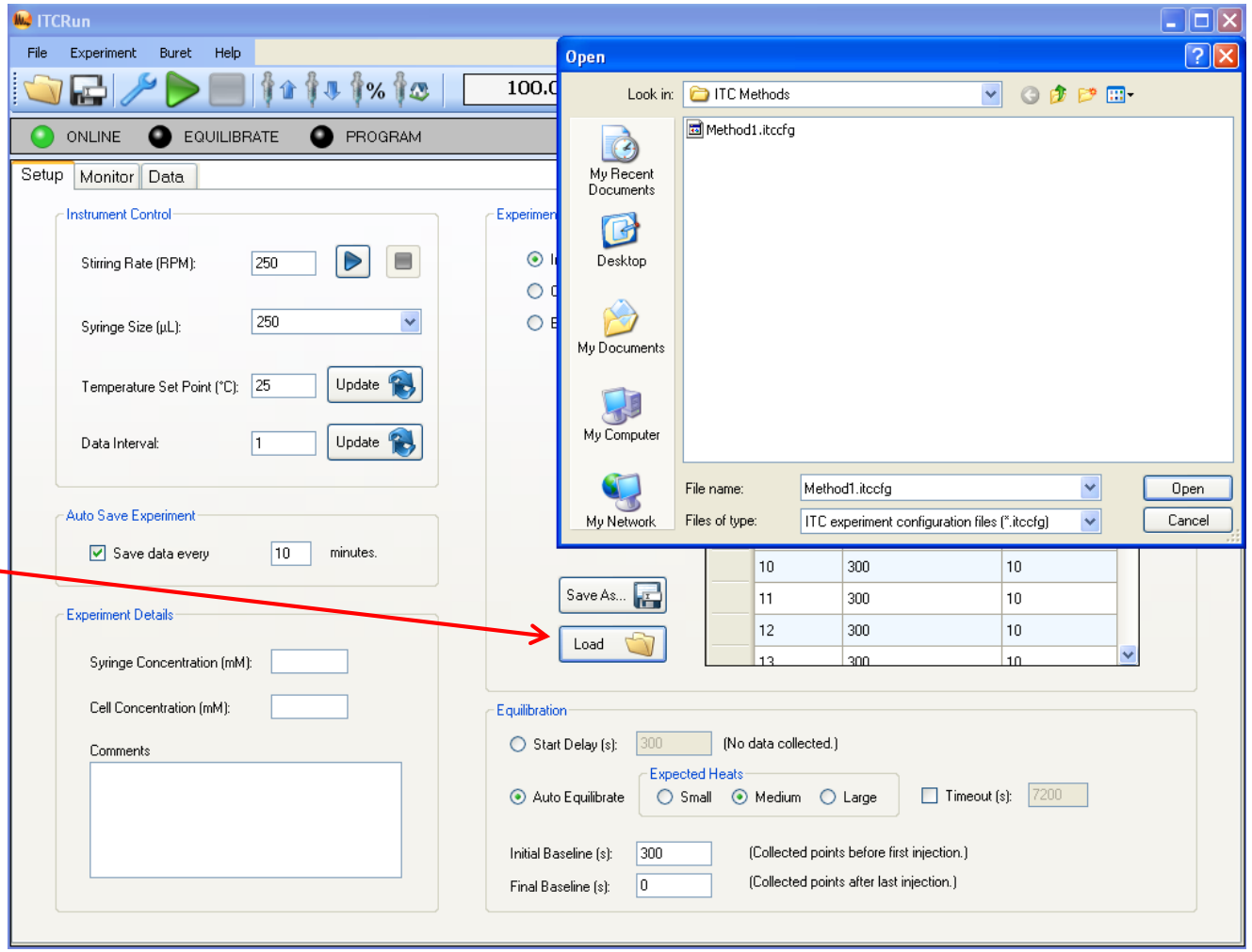
ITCRun Experiment Setup

- **Save the injection parameters (optional)**



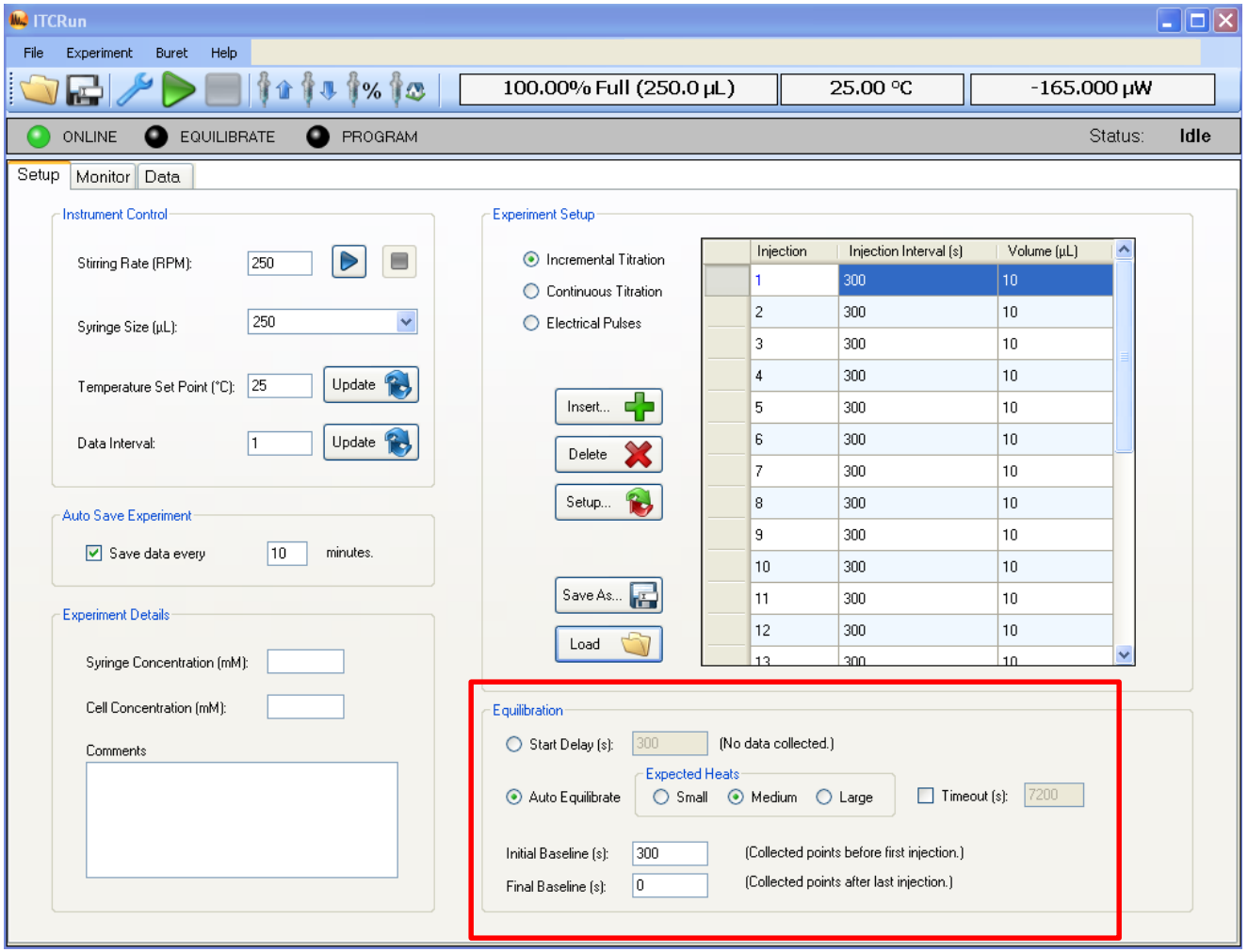
ITCRun Experiment Setup

- **Load previously saved parameters (optional)**



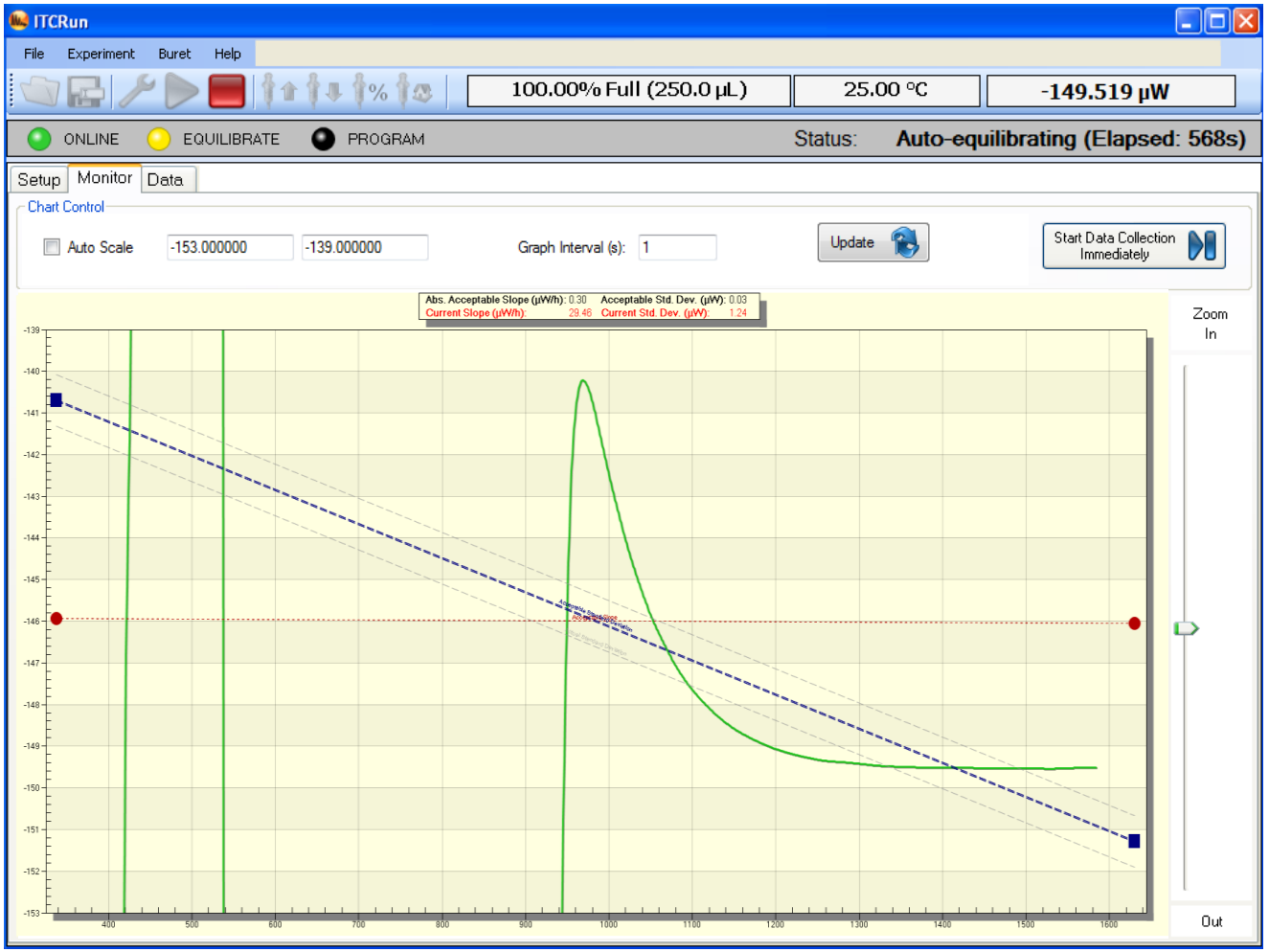
ITCRun Experiment Setup

System/Baseline Auto-Equilibration



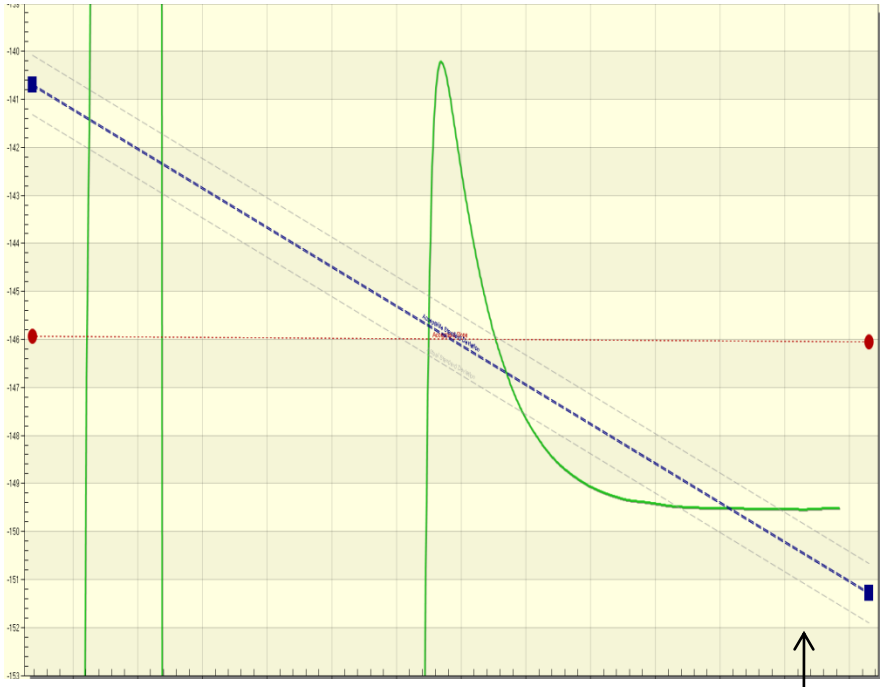
ITCRun Experiment Setup

System/Baseline Auto-Equilibration

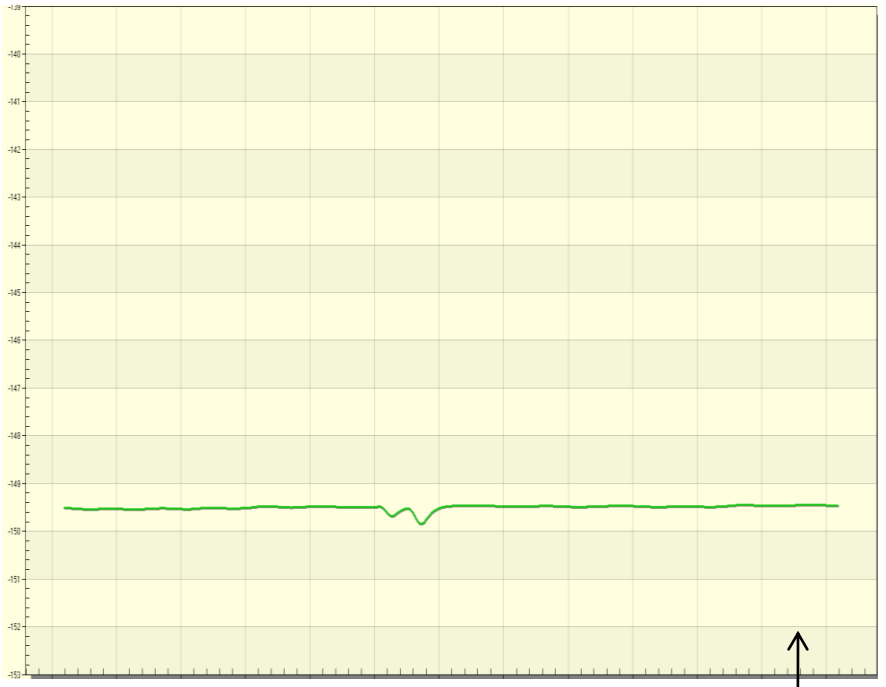


ITCRun Experiment Setup

System/Baseline Auto-Equilibration



~10 minutes



~30 minutes

ITC Data analysis

TA Instruments –Waters LLC

Microcalorimetry Applications

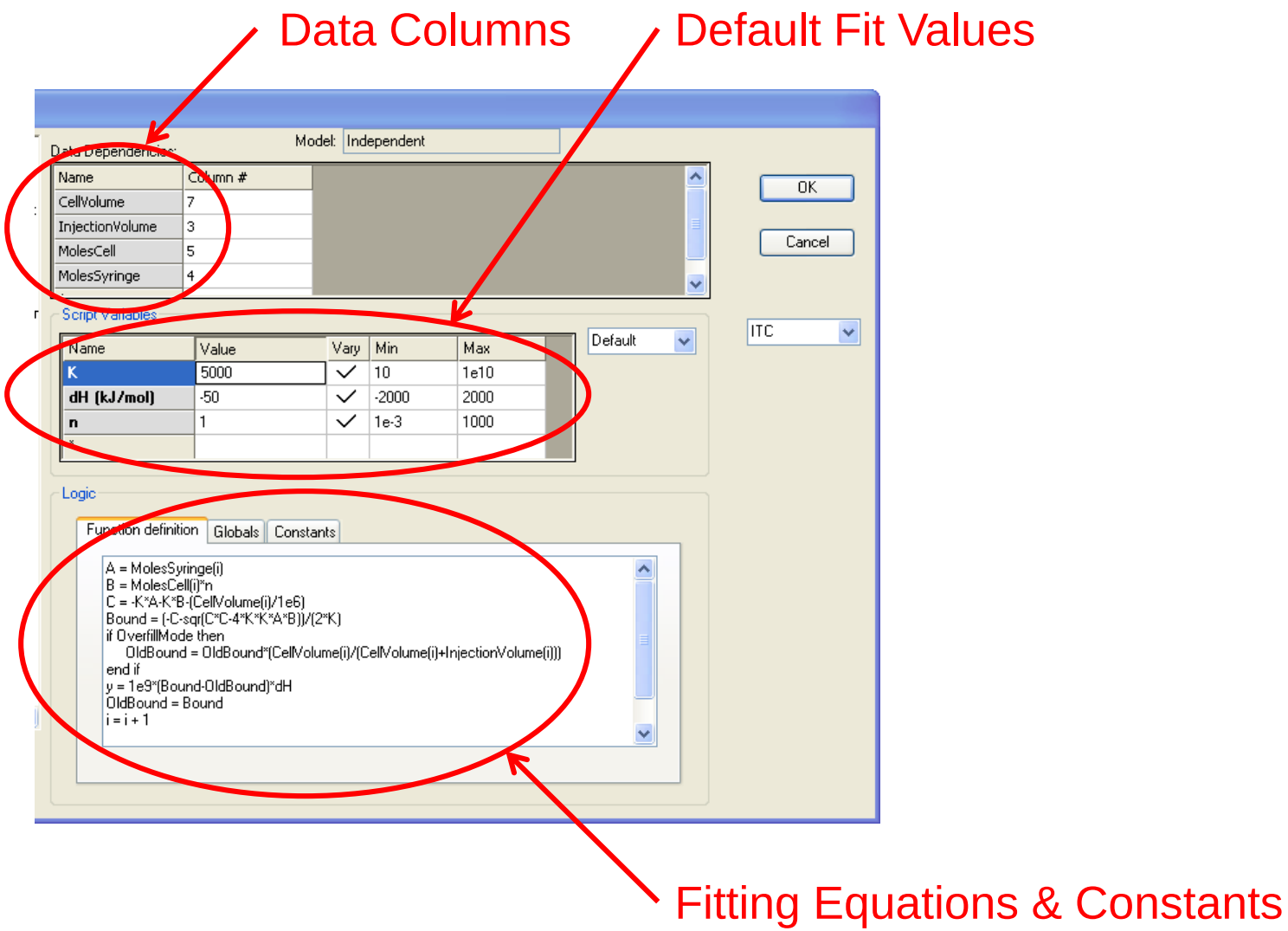
Nano ITC Data Fitting

- Full Suite of ITC data fitting models
  - Independent
  - Minimized Independent
  - Multiple sites
  - Competetive replacement
  - Blank (linear or constant)
  - Dimer dissociation
- Flexible Overlay Graphing
  - Flexible display of multiple graphs
  - User selectable format for graph export
- Statistics on results (K_a, ΔH, n)
  - User adjustable fitting iterations
  - Confidence interval calculations
  - Visual graph of fitting iteration results
  - Statistics on results (K_a, ΔH, n)
- New Report Creation features
  - Integrate text with tables and graphs
  - User selectable format

Model Overview

- **Blank models for fitting**
  - **Blank (constant)**
  - **Blank (linear)**
- **Most common**
  - **Independent model (single sites or multiple equivalent sites)**
  - **Multiple Sites**
  - **Competitive Replacement**
  - **Dimer dissociation**
- **Other models**
  - **Cooperative**
  - **Enthalpy Screening**
  - **Sequential (Two sites)**
  - **Sequential (Three sites)**

NanoAnalyze ITC Fitting Models



Model Overview

- **Blank (constant)** – a constant integer value is fit to the data. This model is often used in conjunction with another fitting model as a background correction factor.
- **Blank (linear)** – the same as the blank (constant) but with a slope and intercept.
- **Independent model** – it models an interaction of “n” ligands with a macromolecule that has one binding site (or multiple equivalent binding sites).
- **Multiple Sites** – this model allows for fitting to two independent sites, each with a unique K, n, and ΔH.
- **Competitive Replacement** – this model allows for fitting a K, n, and ΔH of one ligand while the thermodynamic values for another bound ligand are known. It was designed for measurement of very weak or very tight binding ligands.
- **Dimer dissociation** – this model is used for a dimer dissociation experiment where a concentrated solution of dimer dissociates when it enters a lower concentration environment.

Model Overview

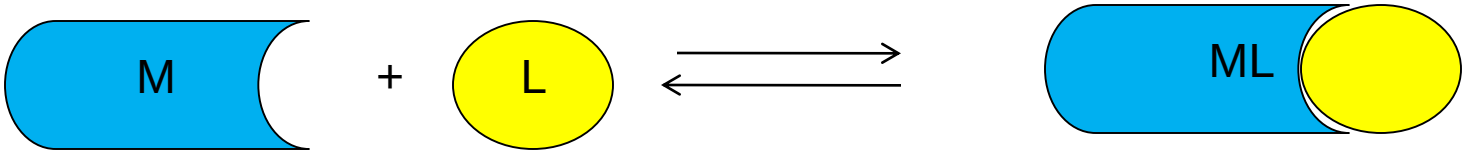
- **Enthalpy Screening** – this is designed to be able to calculate ΔH from a series of data with only the first few injections are done.

- **Sequential (Two sites)** – this is a sequential model with two binding sites where the first binding site has to be populated before the second binding site will accept a ligand.

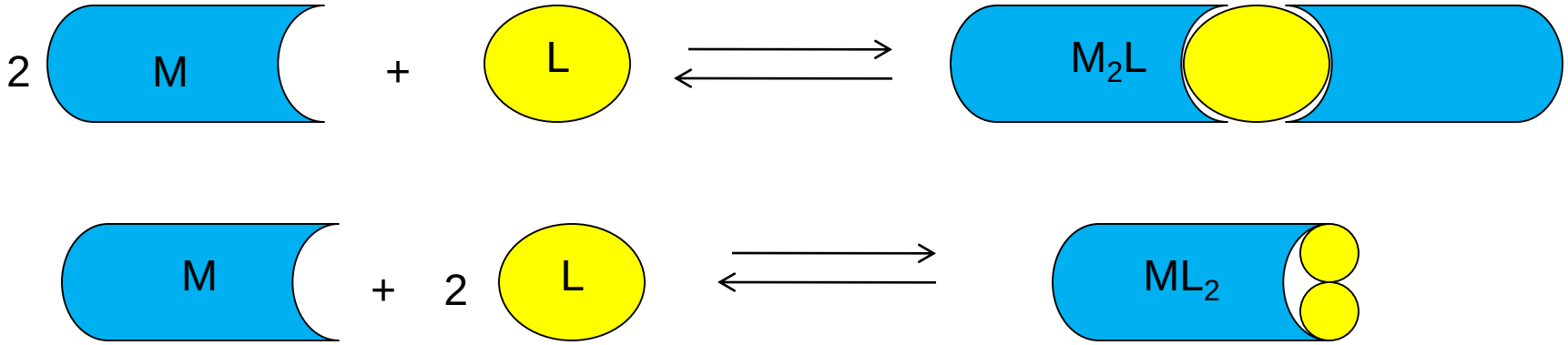
- **Sequential (Three sites)** – similar to the two site above, but there is a third site where both of the first two sites have to be populated before the third site can bind.

Independent Model

- The independent model is a one-site model and can be used for 1:1 interactions.



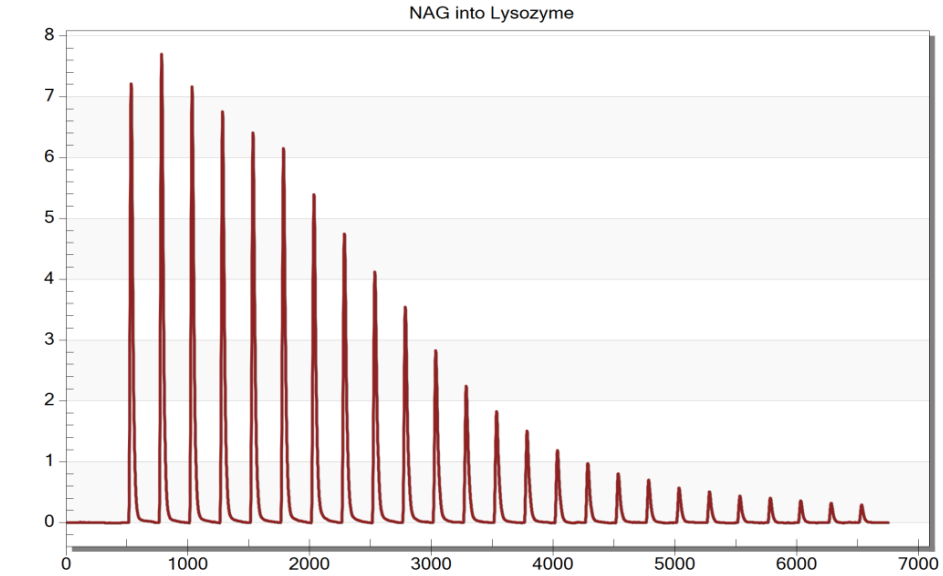
- Higher stoichiometric ratios can also be appropriately fit to this model as long as the association is concerted.

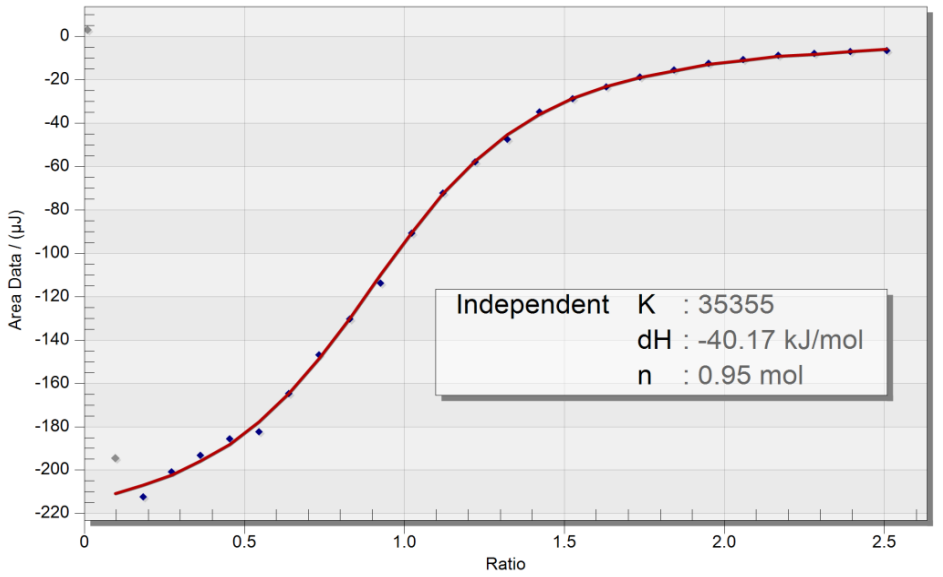


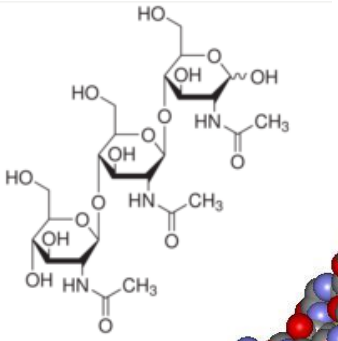
- A binding isotherm for this type of interaction will have a sigmoid shape

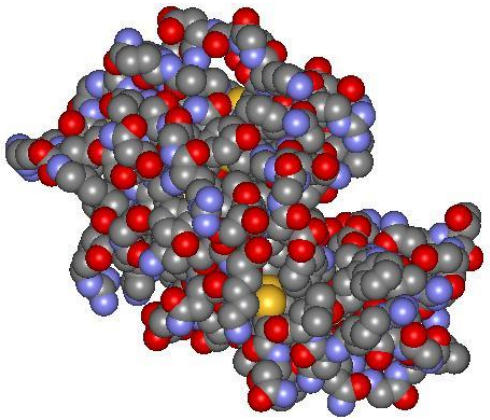
For more information see Freire E.; Mayorga O.; Straume M. “Isothermal Titration Calorimetry” *Analytical Chemistry, 1990 62, 950A-959A*

Independent Model









N,N',N''-triaceytlchitotriose $(NAG)_3$ → Lysozyme

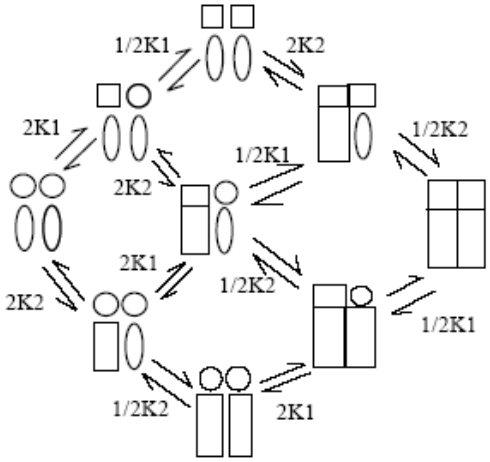
100 mM $Na_2H_2PO_4$ pH 3.0

Multiple Site Model

- The multiple site model is a two-site independent model. The ligand binds two chemically distinct sites on a receptor molecule.

- A binding isotherm for this type of interaction can have two inflections or a single asymmetrical inflection.

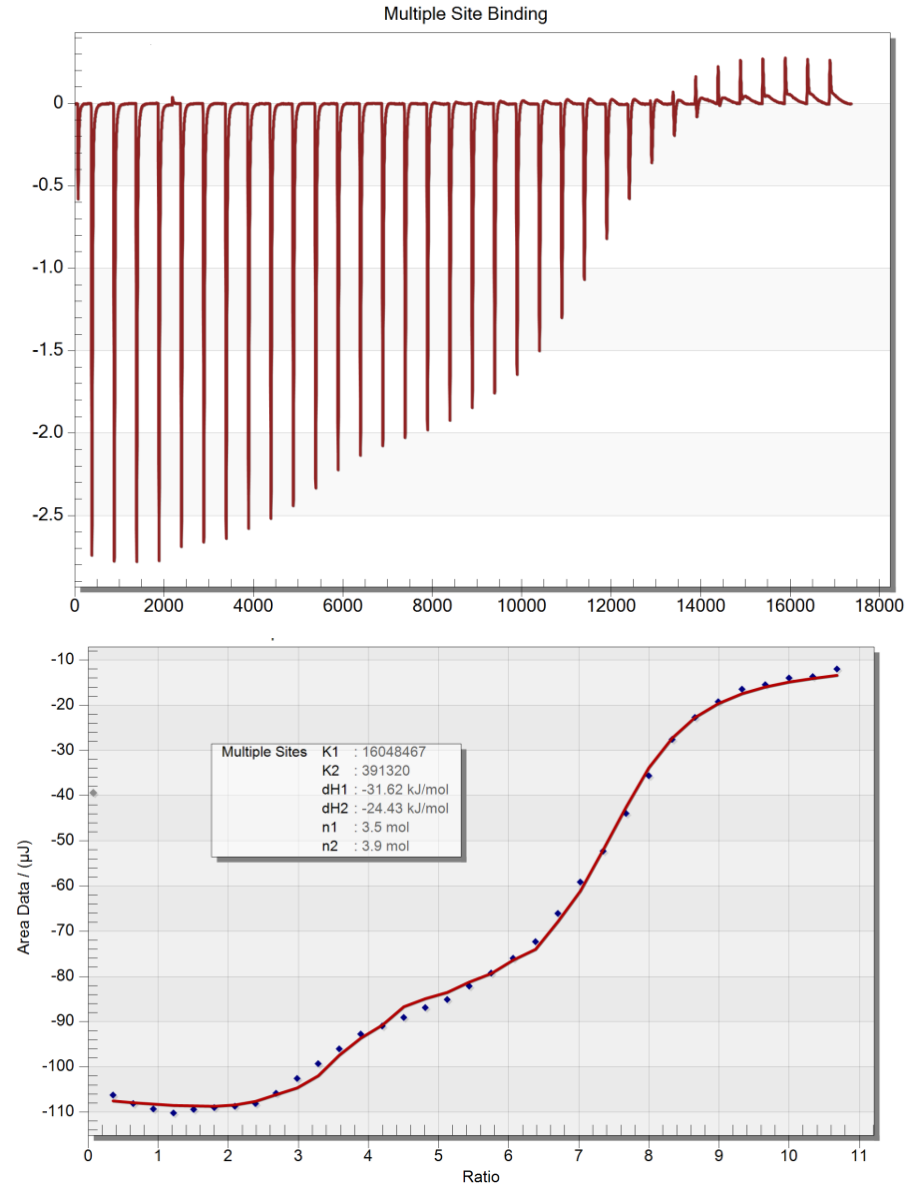
- The shape of the curve depends of on the affinities of the ligand. If the affinities are similar then the isotherm will have a single asymmetrical inflection. If the affinities are several log-orders different, then two inflections could be observed.

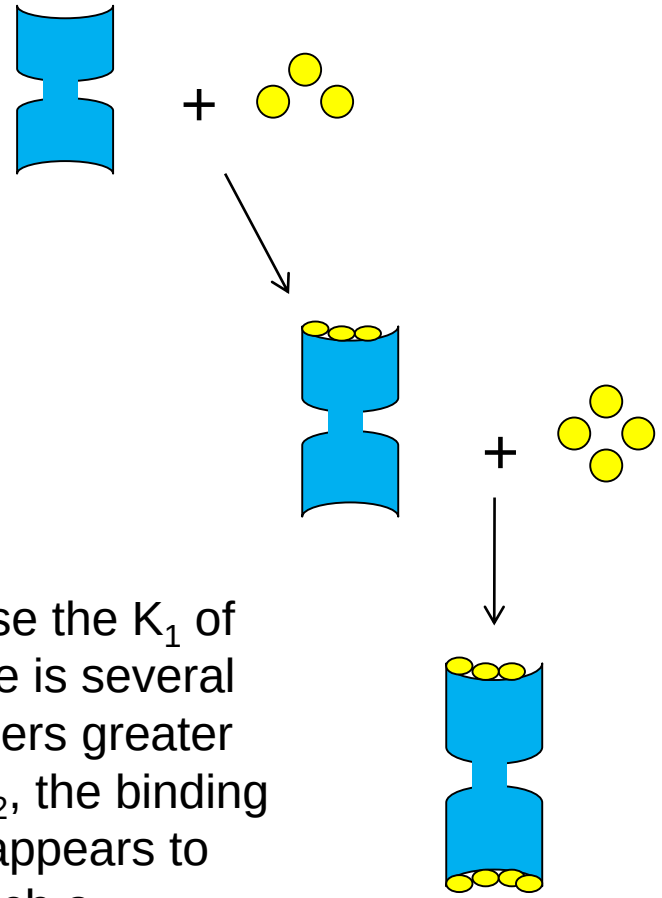


Rectangular regions represent the subunits to which the Ligand is bound.

For more information see Freire E.; Mayorga O.; Straume M.
Analytical Chemistry, 1990, vol. 62, No. 18

Multiple Site protein-ligand interaction





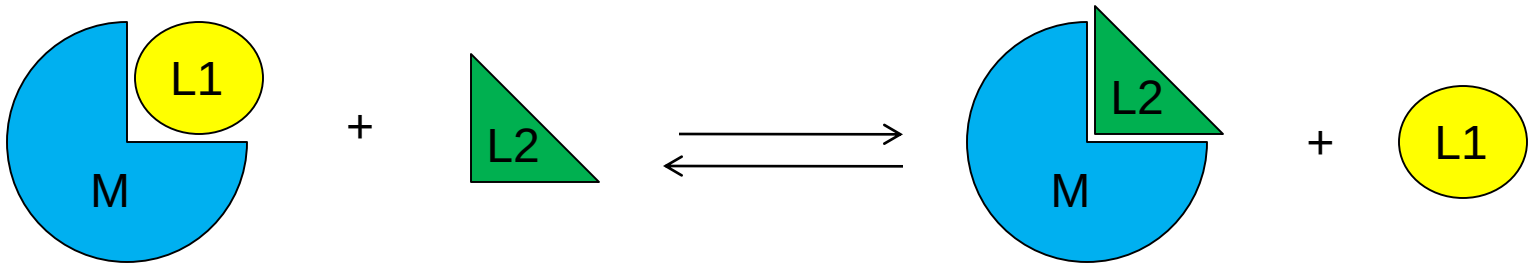
Because the K_1 of one site is several log-orders greater than K_2, the binding event appears to approach a sequential interaction.

Competitive Replacement

- This model allows fitting K, n, and ΔH of one ligand when the thermodynamic values for another bound ligand are known.

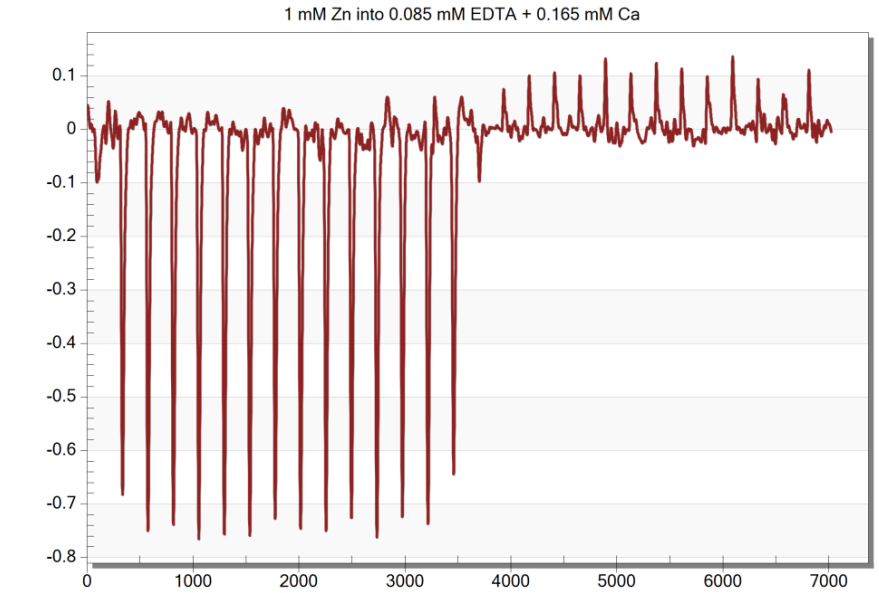
- When setting up the experiment, magnitude of the difference in the K_a values dictate the suggested concentration of the bound ligand, L1. For many cases L1 will need to be in excess of the macromolecule.

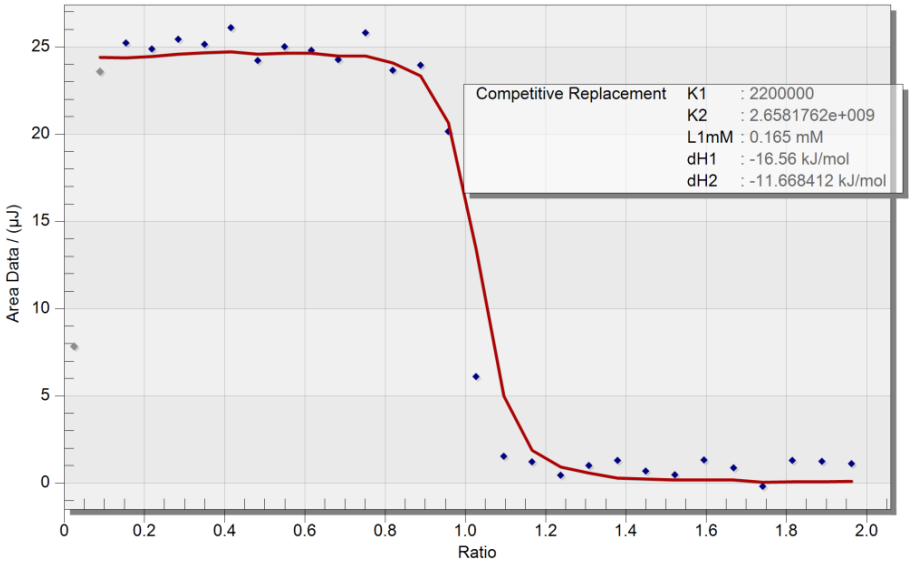
- To determine preliminary experimental conditions it is suggested that a user model the expected results. These conditions can be optimize during experimentation.



For examples and information see Khalifah et al., *Biochemistry* 1993, 32, 3058-3066 and Valazquez Campoy, and Freire *Biophys. Chem*. 2005, 115, 115-124.

Competitive Replacement



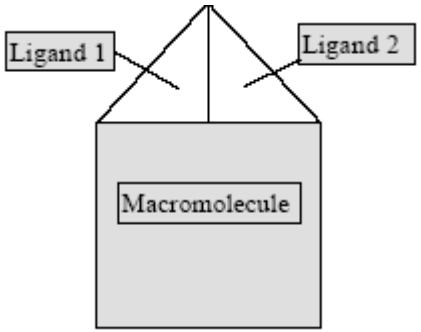


Zn → Ca-EDTA

The thermodynamic values of the weaker binding ligand, L_1, were directly measured.

To fit the data, the K_1, ΔH_1 and L_1 values were inserted and not varied while the fitting algorithm was applied.

Cooperative Model



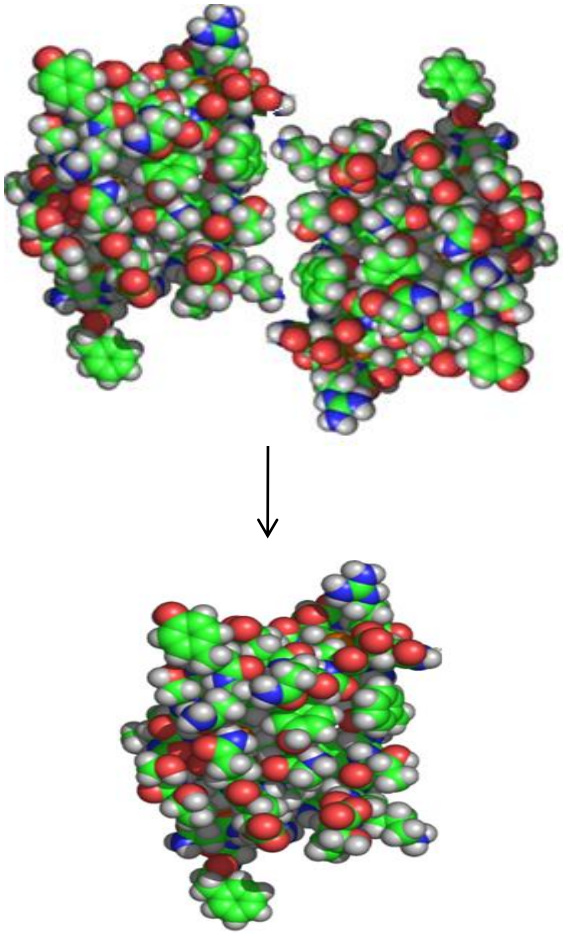
- The cooperative model assumes at least two binding sites on a macromolecule, whereby the binding functions are dependent. For two binding sites the variables that define the cooperative binding are of ΔH and β.

- Typical cooperative interactions can have a “U” shaped binding isotherm; however, the shape of the isotherm is concentration dependent. For example, interactions that have “U” shaped isotherm at one concentration can have sigmoid-shape isotherm at lower concentrations.

- Or fit with the multiple site and apply the Hill equation
  - n(Hill) = 2/(1+((K1/(2*K2))^(1/2))
  - n(Hill) > 1 = cooperative

For more details refer to J. Wyman, S. J. Gill *Binding and Linkage. University* Science Books 1990.

Dimer Dissociation

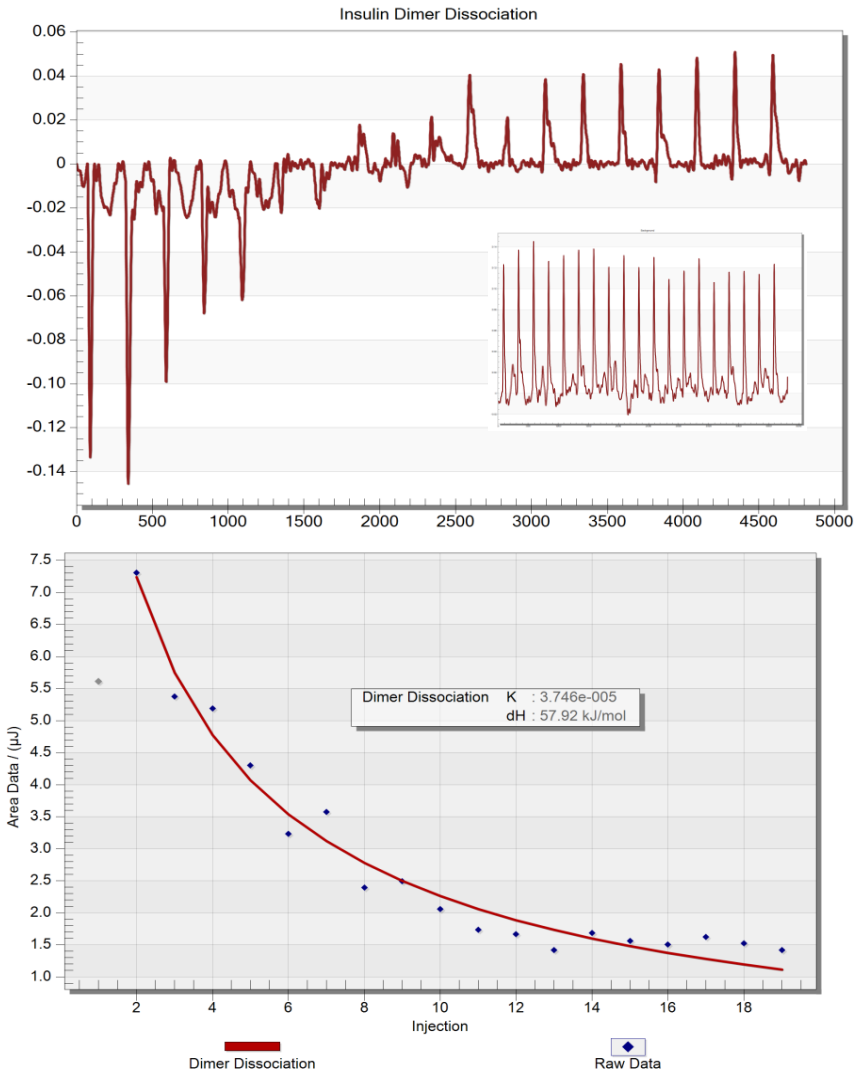


Human Insulin

The dimer dissociation model is used to study dissociation events. The K expressed the K_d (dissociation constant)

Experimentally, a concentrated solution of dimer dissociates when it enters the cell that contains buffer.

Dimer Dissociation



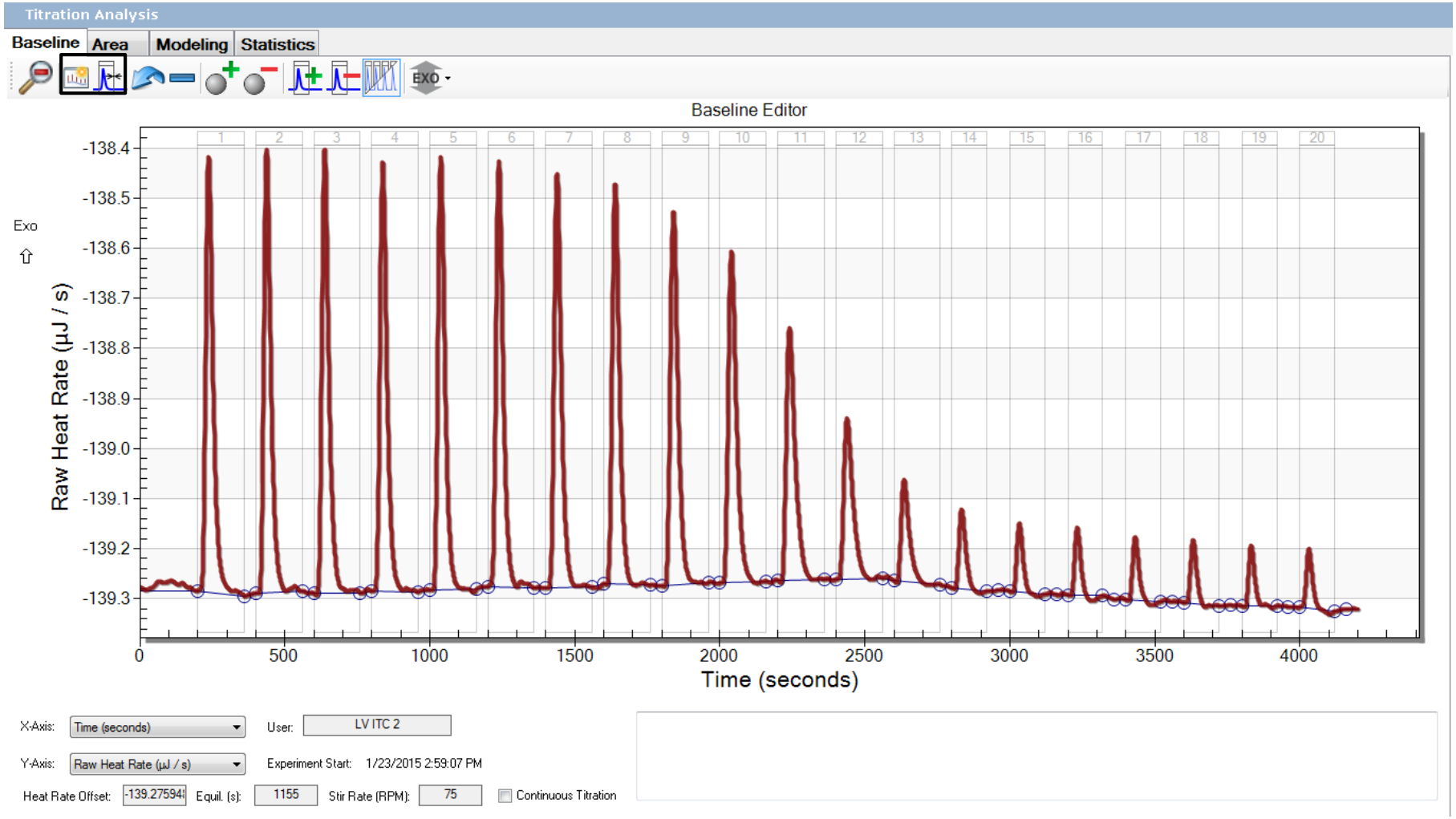
Top Figure is the raw data, inset is background of buffer into buffer and the bottom figure is the integrated, normalized, and fit data.

For 0.2 mM human insulin at pH 2.8 the Kd = 37 µM and Δ H = 58 kJ/mol

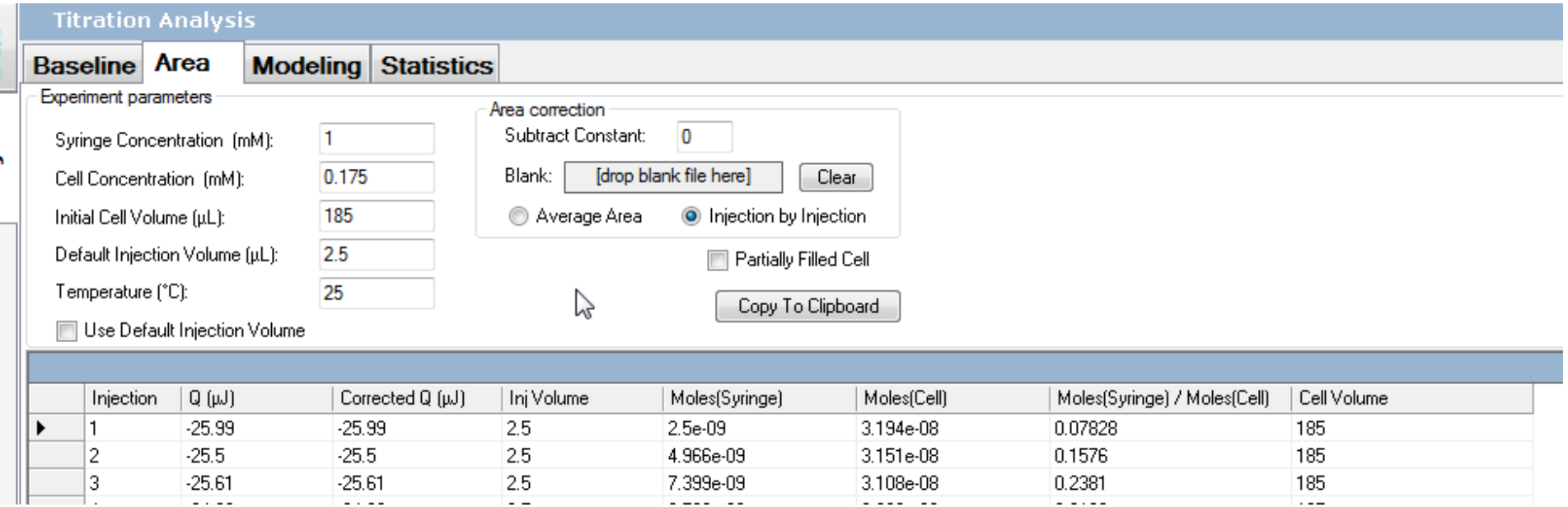
This data are consistent with the ITC literature values for bovine insulin collected at pH 2.5
(K_d= 14 µM and ΔH = 41 kJ/mol)
Lovatt, Cooper, Camilleri, Eur Biophys. J. (1996) 24:354-357

Because of differences in the conditions such as pH, as mentioned in the Lovatt paper, and the type of insulin, bovine versus human, the thermodynamic values are expected to deviate slightly from each other.

NanoAnalyze – Baseline & Integration Settings

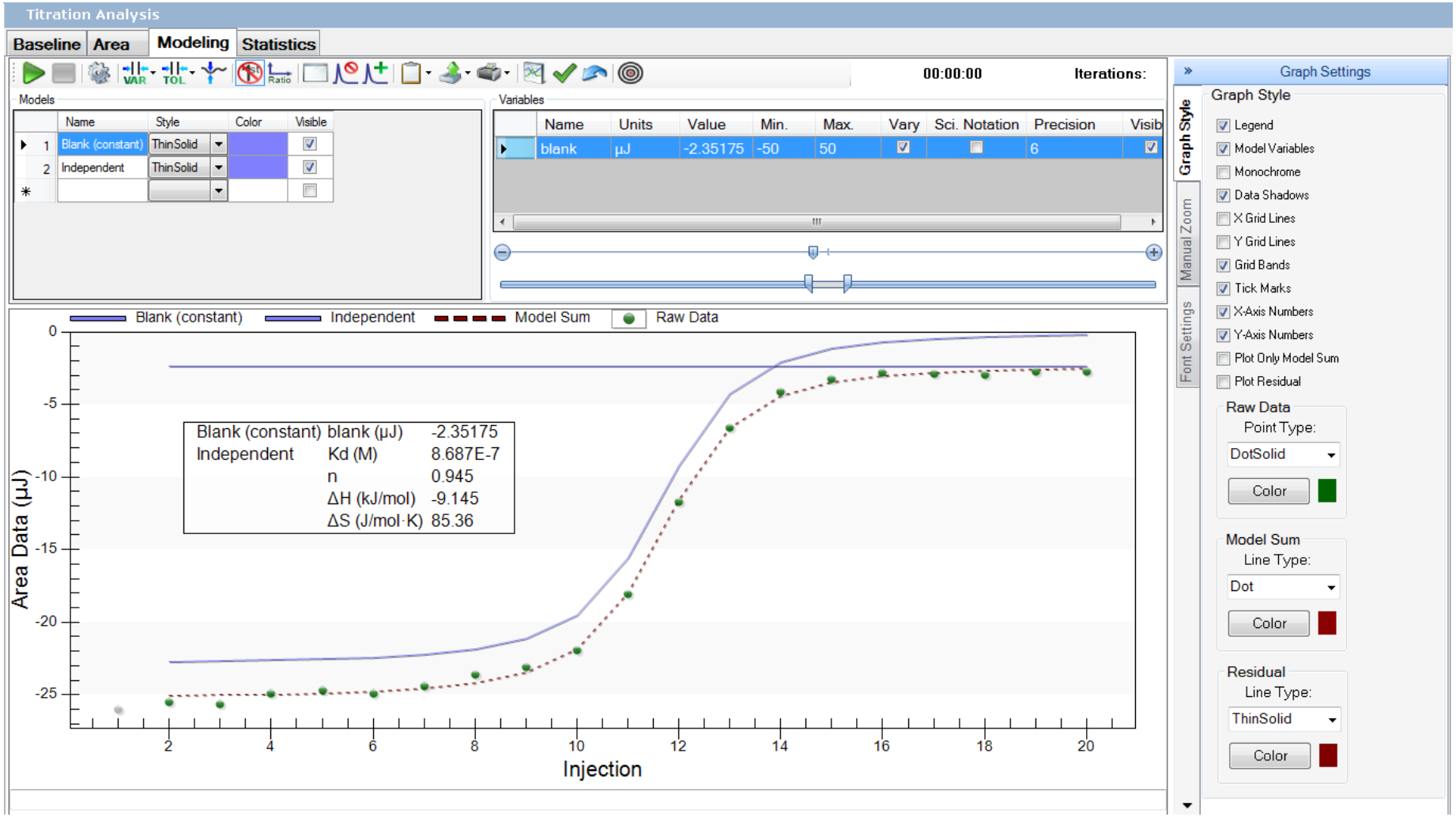


NanoAnalyze – Area Correction, Volumes, Moles

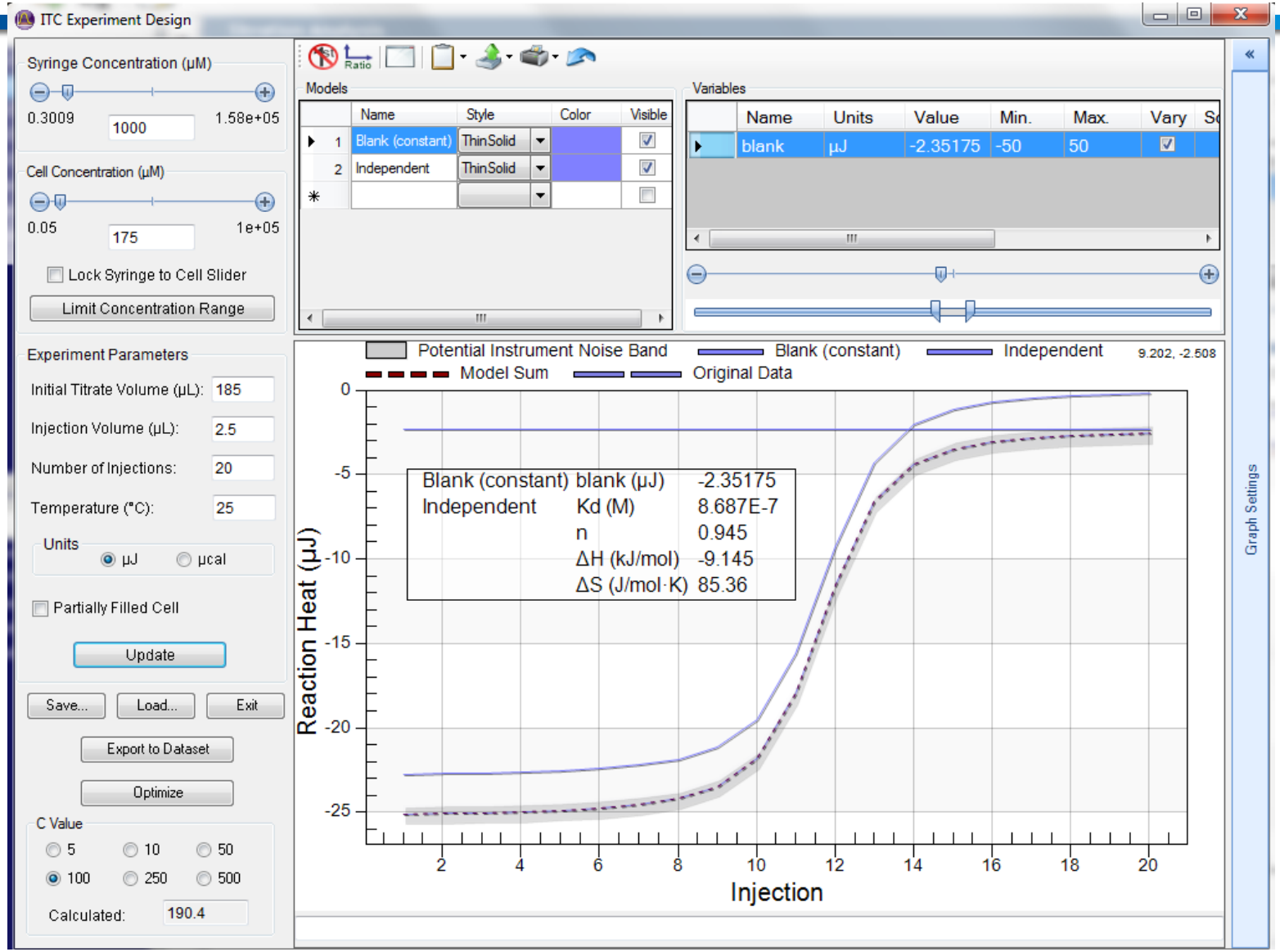


Injection	Q (µJ)	Corrected Q (µJ)	Inj Volume	Moles(Syringe)	Moles(Cell)	Moles(Syringe) / Moles(Cell)	Cell Volume
1	-25.99	-25.99	2.5	2.5e-09	3.194e-08	0.07828	185
2	-25.5	-25.5	2.5	4.966e-09	3.151e-08	0.1576	185
3	-25.61	-25.61	2.5	7.399e-09	3.108e-08	0.2381	185

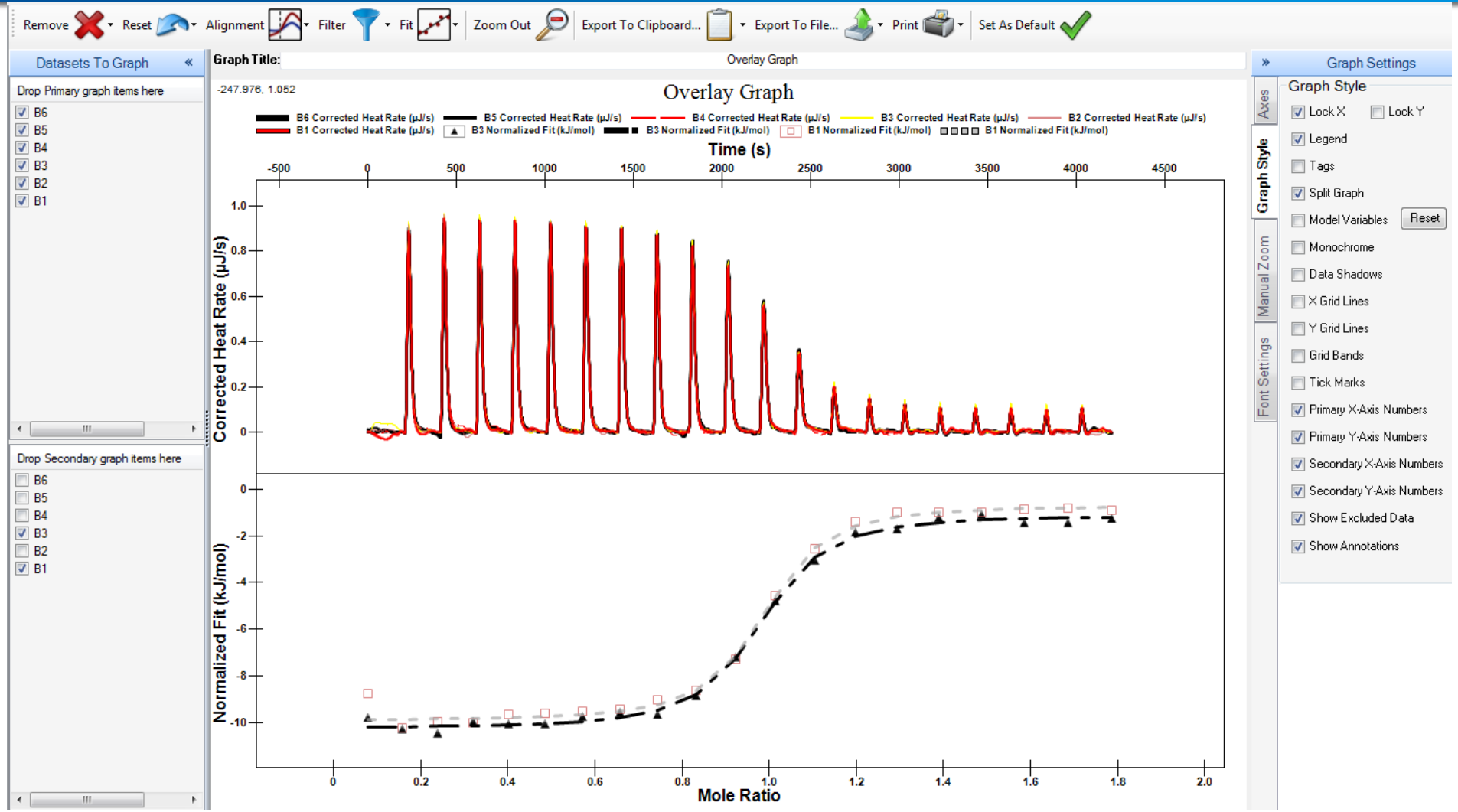
NanoAnalyze – Data Modeling



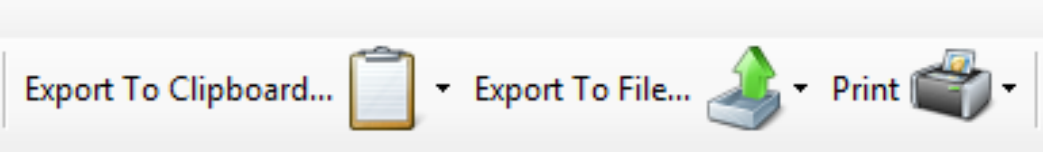
Optimization to Experimental Design



NanoAnalyze - Multiple Graph Display



NanoAnalyze – Graph & Data Export Options



- 5 options for image export
- Easy data export to spreadsheet format

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Thank You

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