# Isothermal titration calorimetry: Principles and experimental design

# Agenda

Overview of Isothermal Titration Calorimetry

ITC experimental design

Data analysis

Troubleshooting

# What is isothermal titration calorimetry (ITC)

A direct measurement of the heat generated or absorbed when molecules interact

# Microcalorimetry offers enhanced information content

Label-free

In-solution

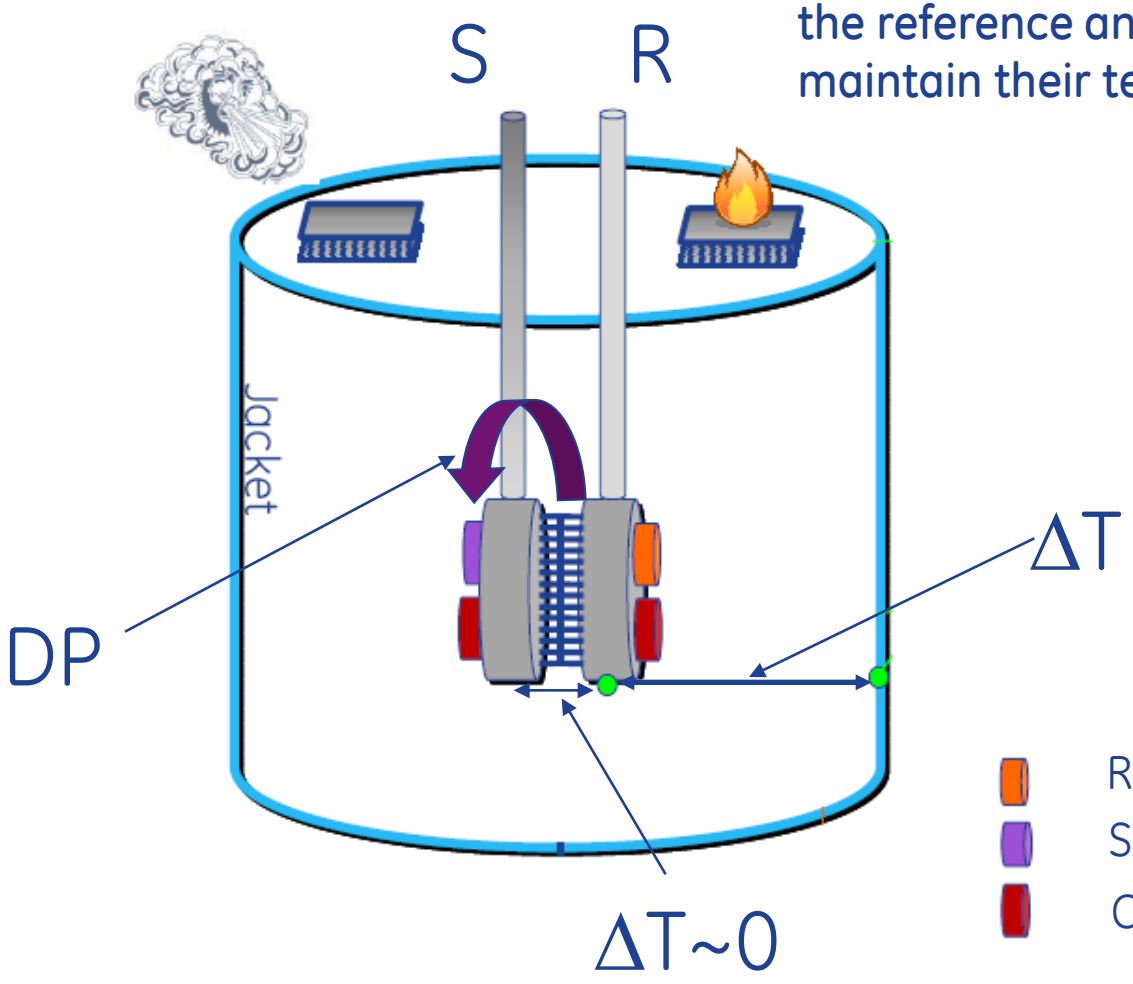
No molecular weight limitations

Optical clarity unimportant

Minimal or no assay development

# How Do ITCs Work?

The DP is a measured power differential between the reference and sample cells necessary to maintain their temperate difference at close to zero




- Reference Calibration Heater
- Sample Calibration Heater
- Cell Main Heater

# Performing an ITC experiment

Ligand in syringe

Macromolecule in sample cell

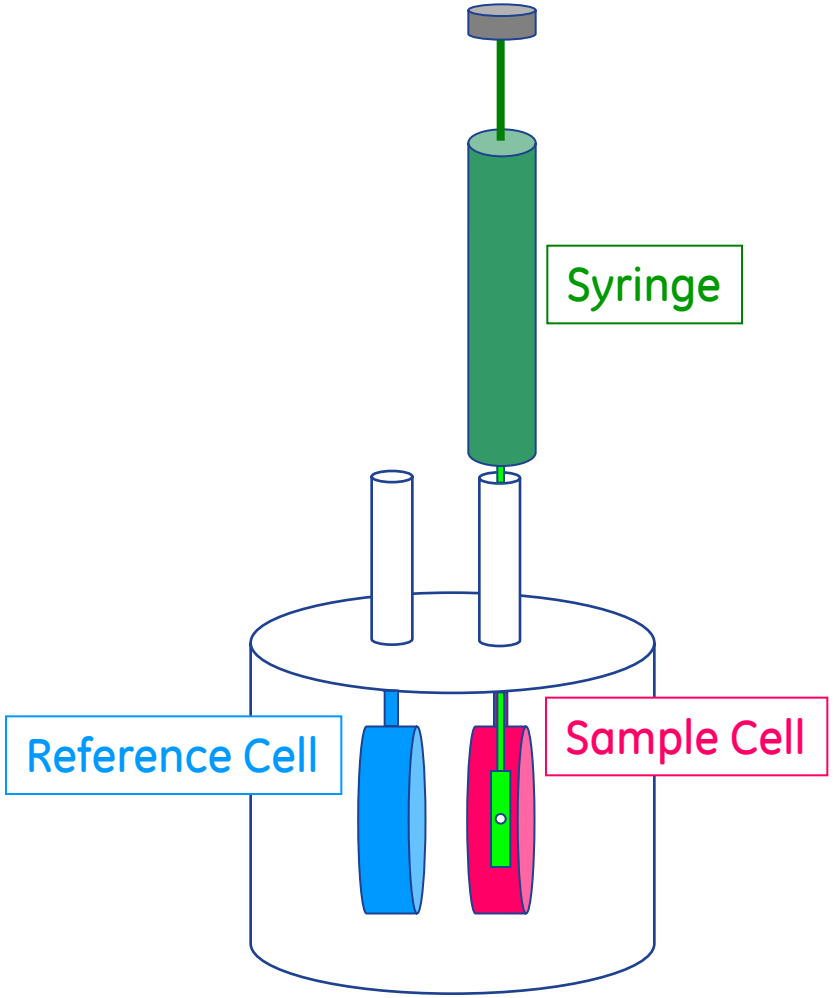
Heat of interaction is measured

Parameters measured from a single ITC experiment:

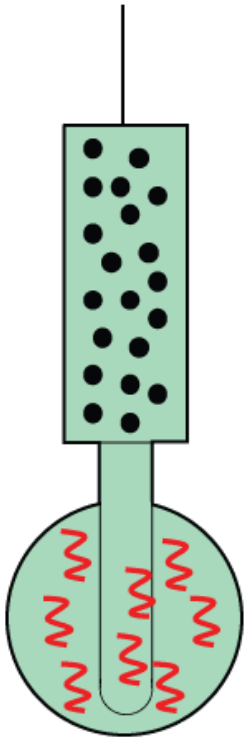
Affinity - $K_D$

Energy (Enthalpy) - $\Delta H$

Number of binding sites - n

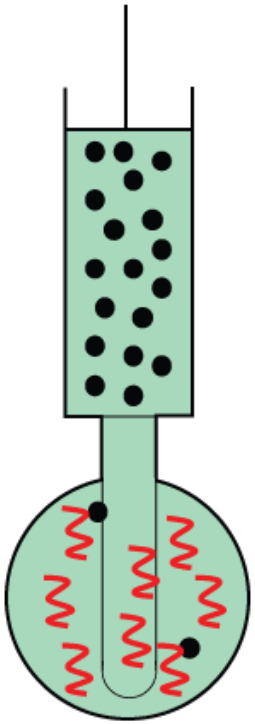


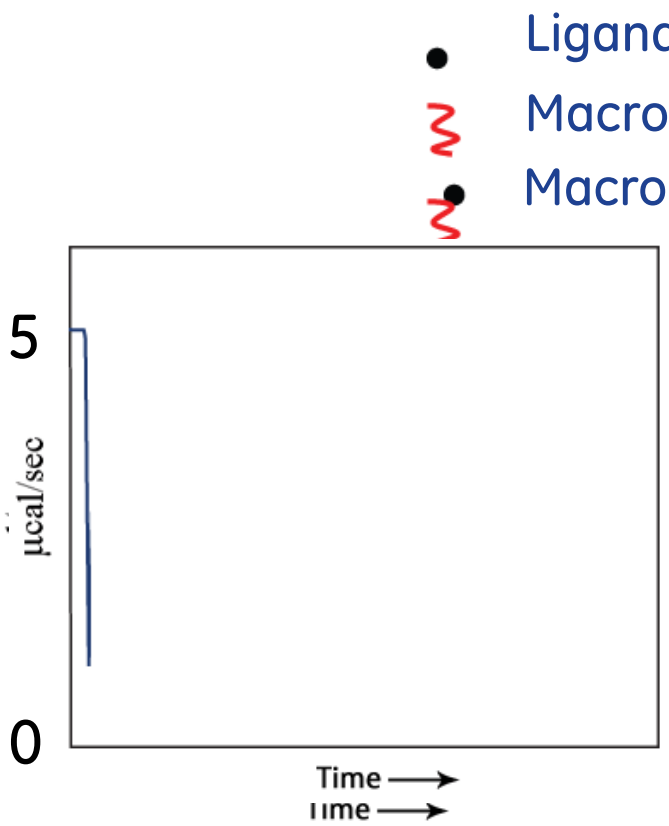
# ITC – Before titration



- Ligand – in syringe
- Macromolecule in ITC cell

# Titration begins: First injection





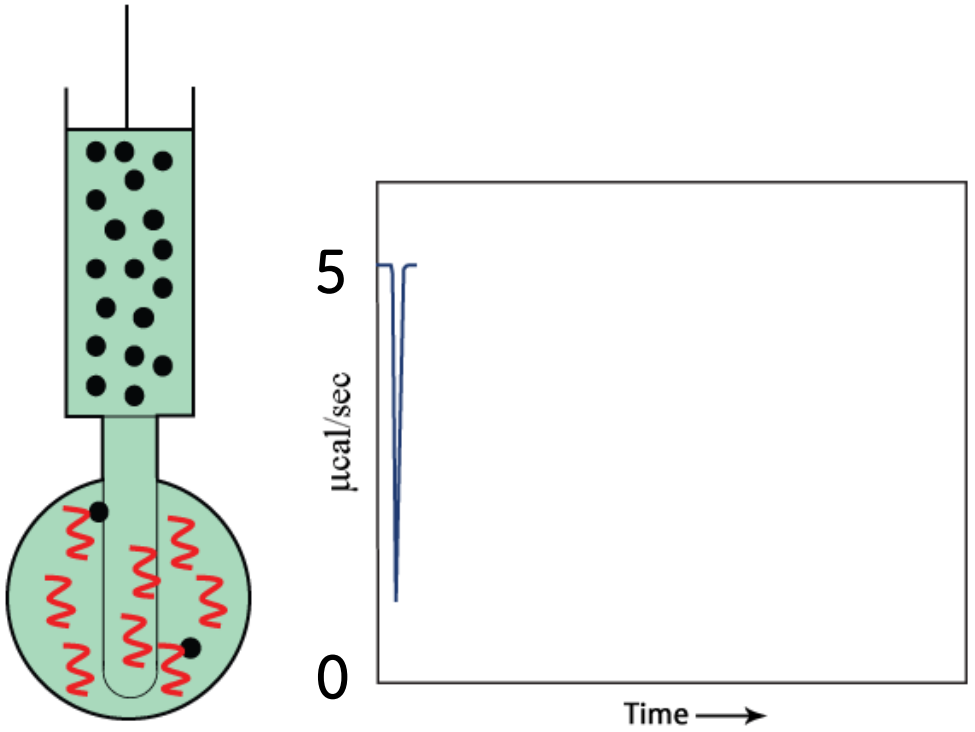

Ligand in syringe

Macromolecule in cell

Macromolecule-ligand complex

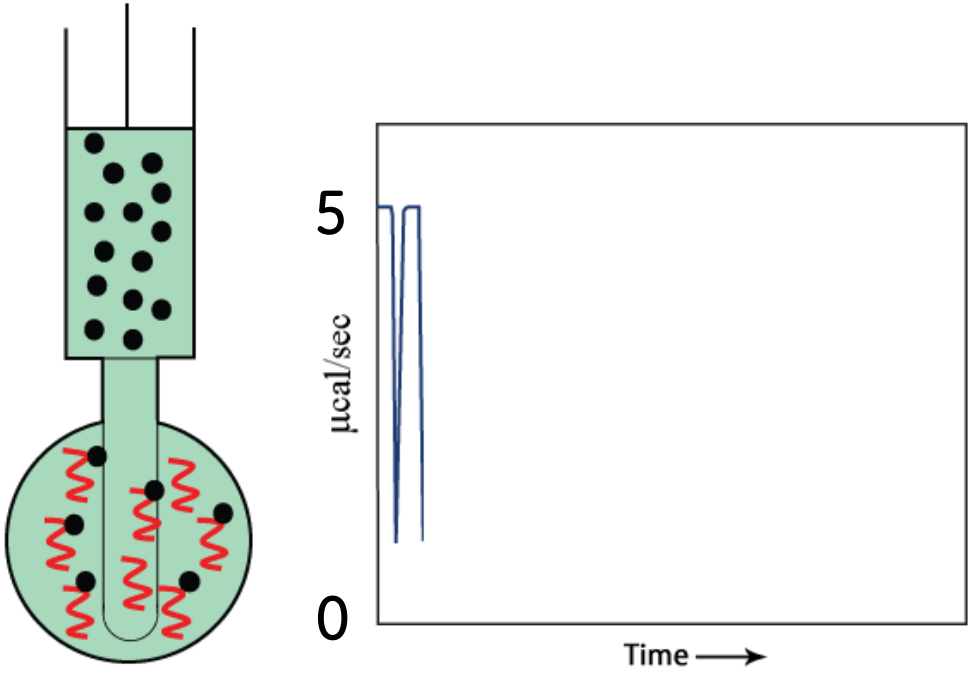
As the first injection is made, all injected ligand is bound to target macromolecule.

# Return to baseline



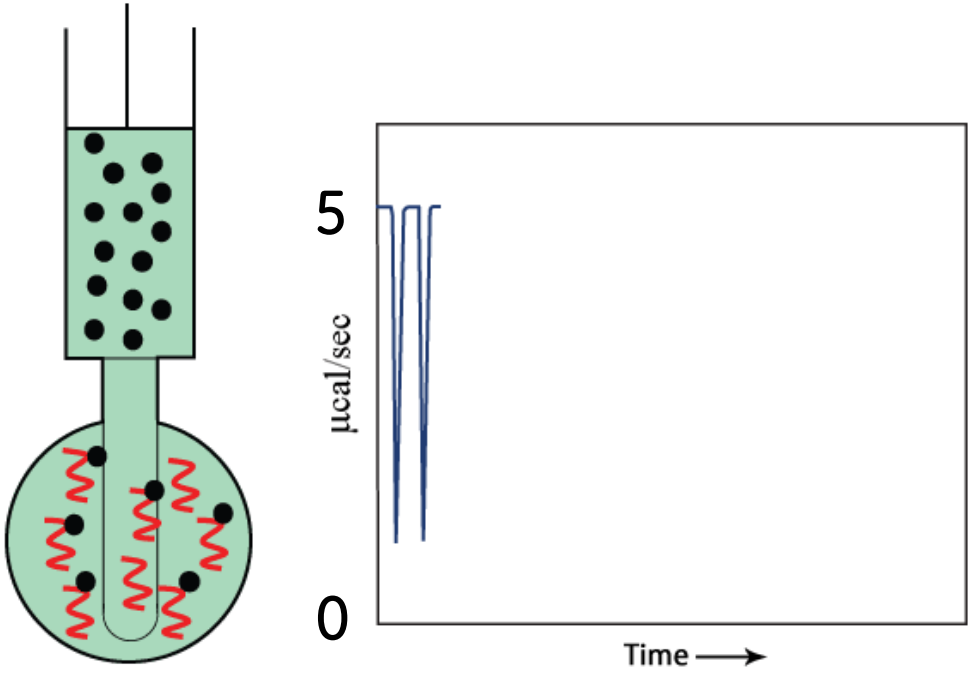

The signal returns to baseline before the next injection.

# Second injection



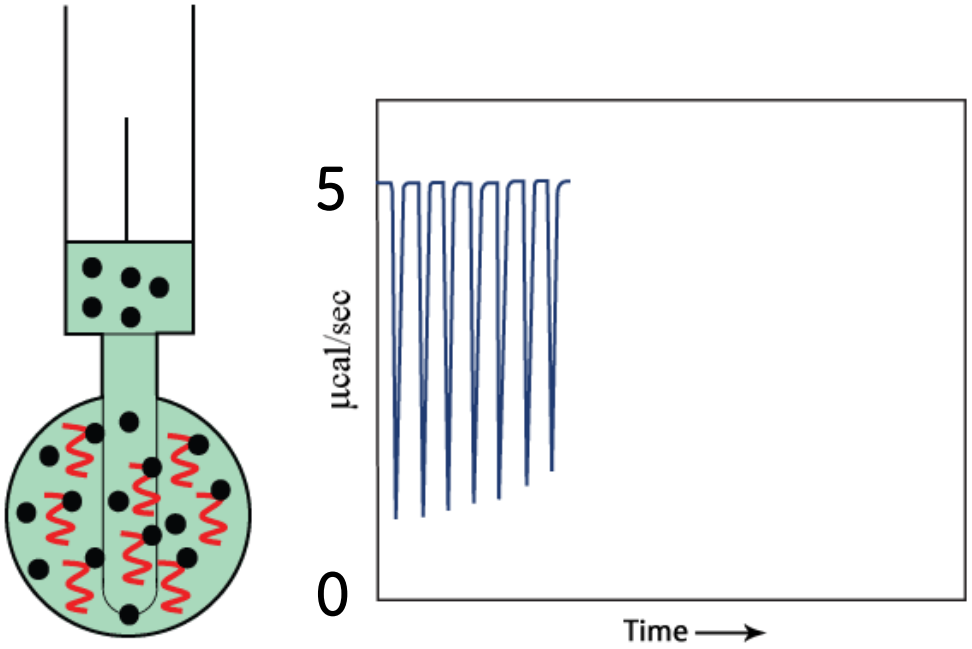

As a second injection is made, again all injected ligand becomes bound to the target.

# Second return to baseline



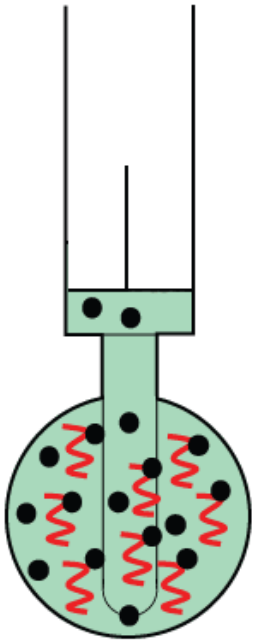

Signal again returns to baseline before next injection.

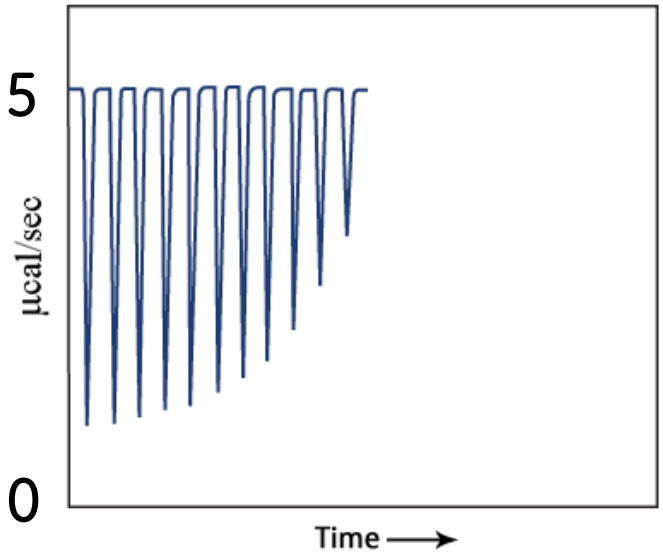
# Injections continue




As the injections continue, the target becomes saturated with ligand, so less binding occurs and the heat change starts to decrease.

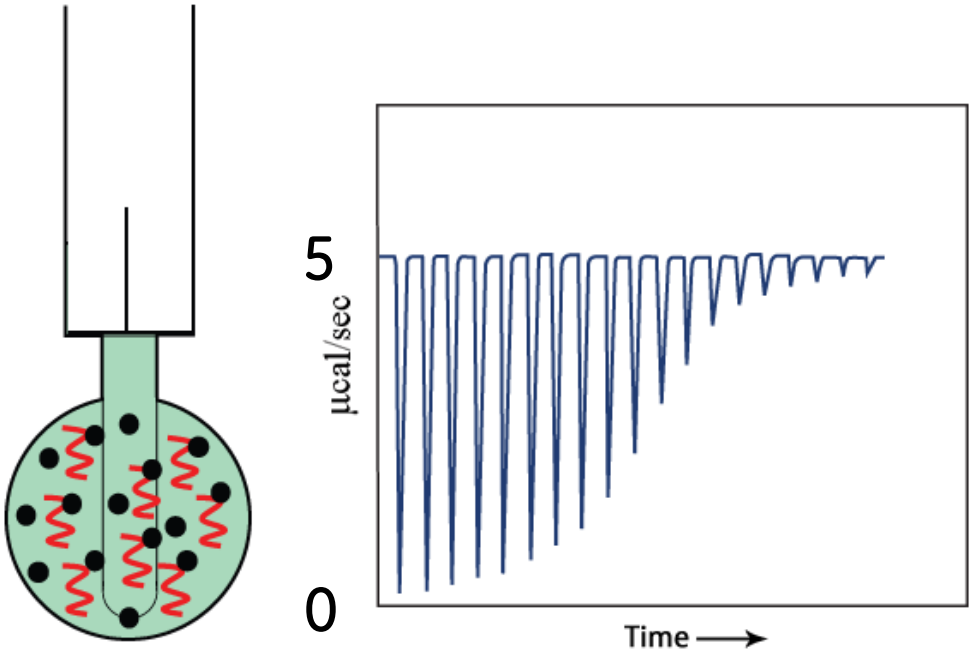
# Injections continue





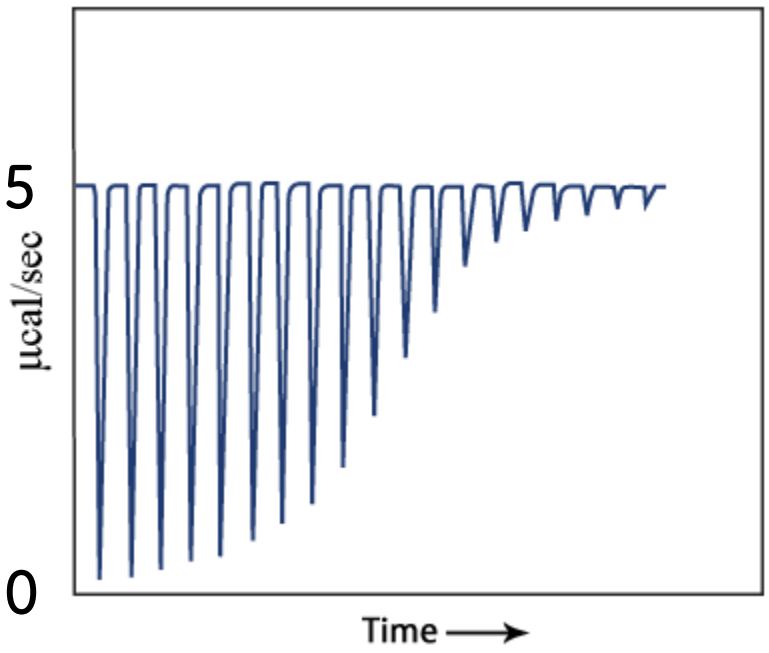

As the injections continue, the target becomes saturated with ligand so less binding occurs and the heat change starts to decrease.

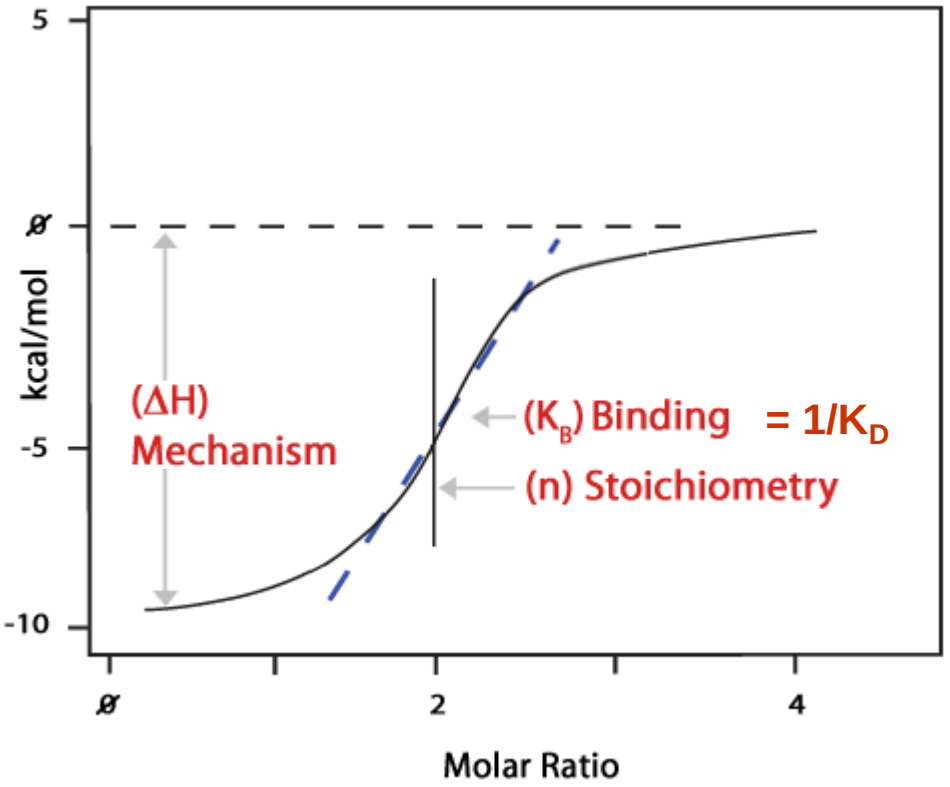
# End of titration



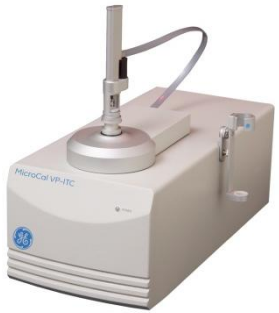

When the macromolecule is saturated with ligand, no more binding occurs, and only heat of dilution is observed.

# Experimental results



# MicroCal™ ITC systems

| MicroCal™ VP-ITC | MicroCal™ iTC$_{200}$ | MicroCal™ Auto-iTC$_{200}$ |
|---|---|---|
| • 1400 μL cell<br>• Manual sample loading<br>• Up to 5 samples/day | • Sensitive<br>• Fast<br>• Easy to use<br>• $K_D$ from mM to nM<br>• 200 μL cell<br>• Upgradable to full automation | • Unattended operation<br>• Up to 75 samples/day *(using single injection method)*<br>• $K_D$ from mM to nM<br>• Sample cell is 200 μL<br>• Easy to use<br>• 96-well plate format |
|  |  |  |

# Why ITC?

# Heat is a fundamental natural property…

A single titration can yield information on:

Overall binding affinity

Hydrogen bonds and van der Waals interactions

Hydrophobic and conformational effects

Stoichiometry

calorimetry is a direct readout

# Stoichiometry

Number of ligand binding sites per macromolecule

If one binding site the stoichiometry is 1

By convention a “Ligand” has one binding site

A “Macromolecule” can have more than one binding site

# Effective Binding Affinity Range

$K_D$ in mM to nM range

Weak binding – low C-value method

Tight binding - minimize injection volume and concentration or use competitive (displacement) binding procedure and fitting model

# Thermodynamics

$K_B$ – binding constant

$$K_D = 1/K_B = \frac{[L] \times [M]}{[ML]}$$

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

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

# Free energy change

- $\Delta G$ is change in free energy
- $\Delta G \leq 0$ for spontaneous process
- More negative $\Delta G$, higher affinity

# Enthalpy change

- $\Delta H$ – measure of the energy content of the bonds broken and created. The dominant contribution is from hydrogen bonds.
- Negative value indicates enthalpy change favoring the binding
- Solvents play a role

# Entropy change

- $\Delta S$ – positive for entropically driven reactions
- Hydrophobic interactions
- Solvation entropy (favorable) due to release of water
- Conformational degrees of freedom (unfavorable)

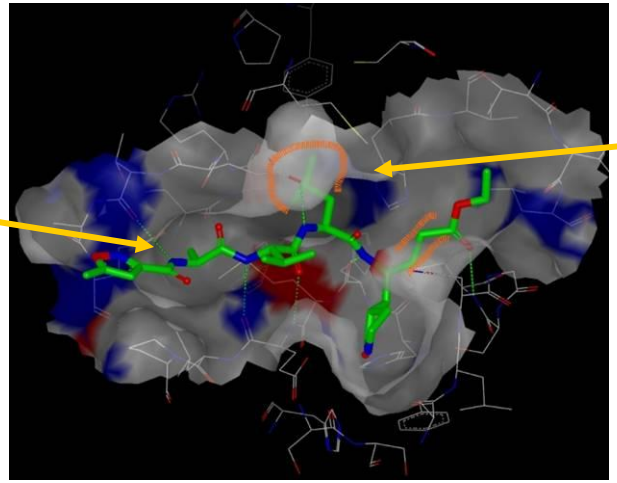
# Microcalorimetry provides a total picture of binding energetics

Overall binding affinity $K_D$ correlates with $IC_{50}$ or $EC_{50}$.
This is directly related to $\Delta G$, the total free binding energy

$\Delta H$, enthalpy is indication of changes in hydrogen and van der Waals bonding

$-T\Delta S$, entropy is indication of changes in hydrophobic interaction and conformational changes

N, stoichiometry indicates the ratio of ligand-to-macromolecule binding



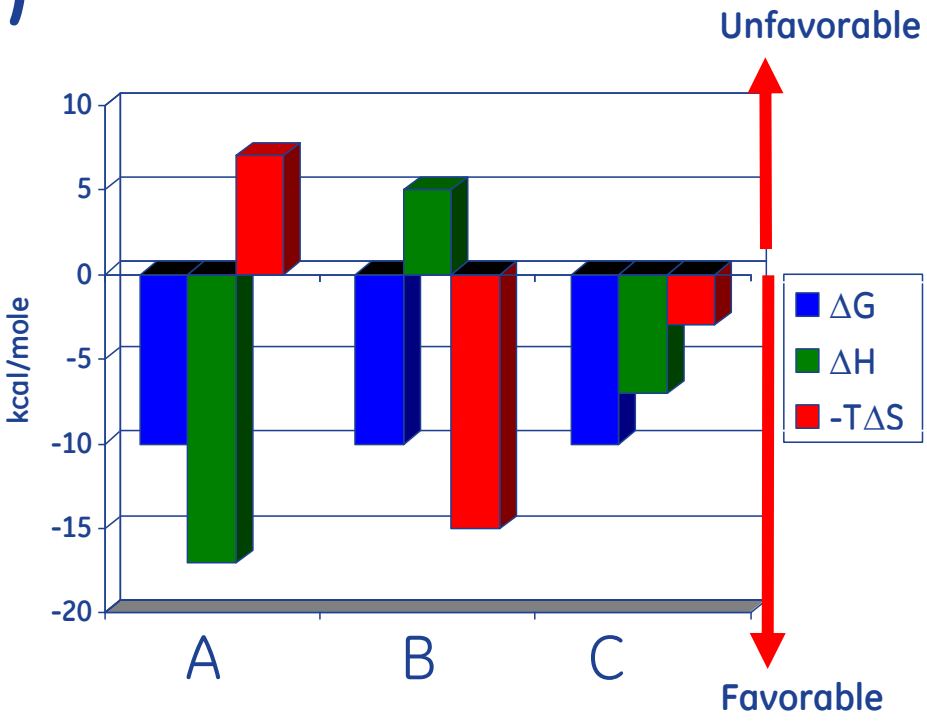

$\Delta G = \Delta H - T\Delta S$

# 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

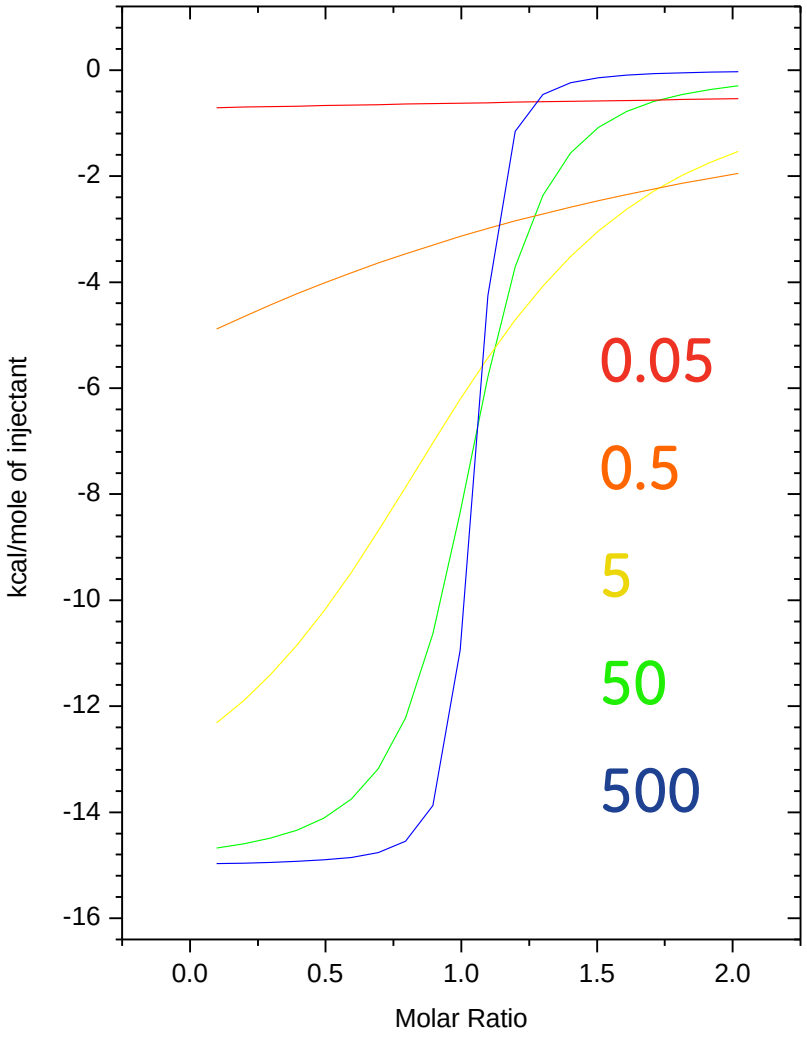
C. Favorable hydrogen bonds and hydrophobic interaction




ITC results are used to get insights into mechanism of binding

# How to get good ITC data

# C Values




$$C = \frac{[M]}{K_D}$$

Example:

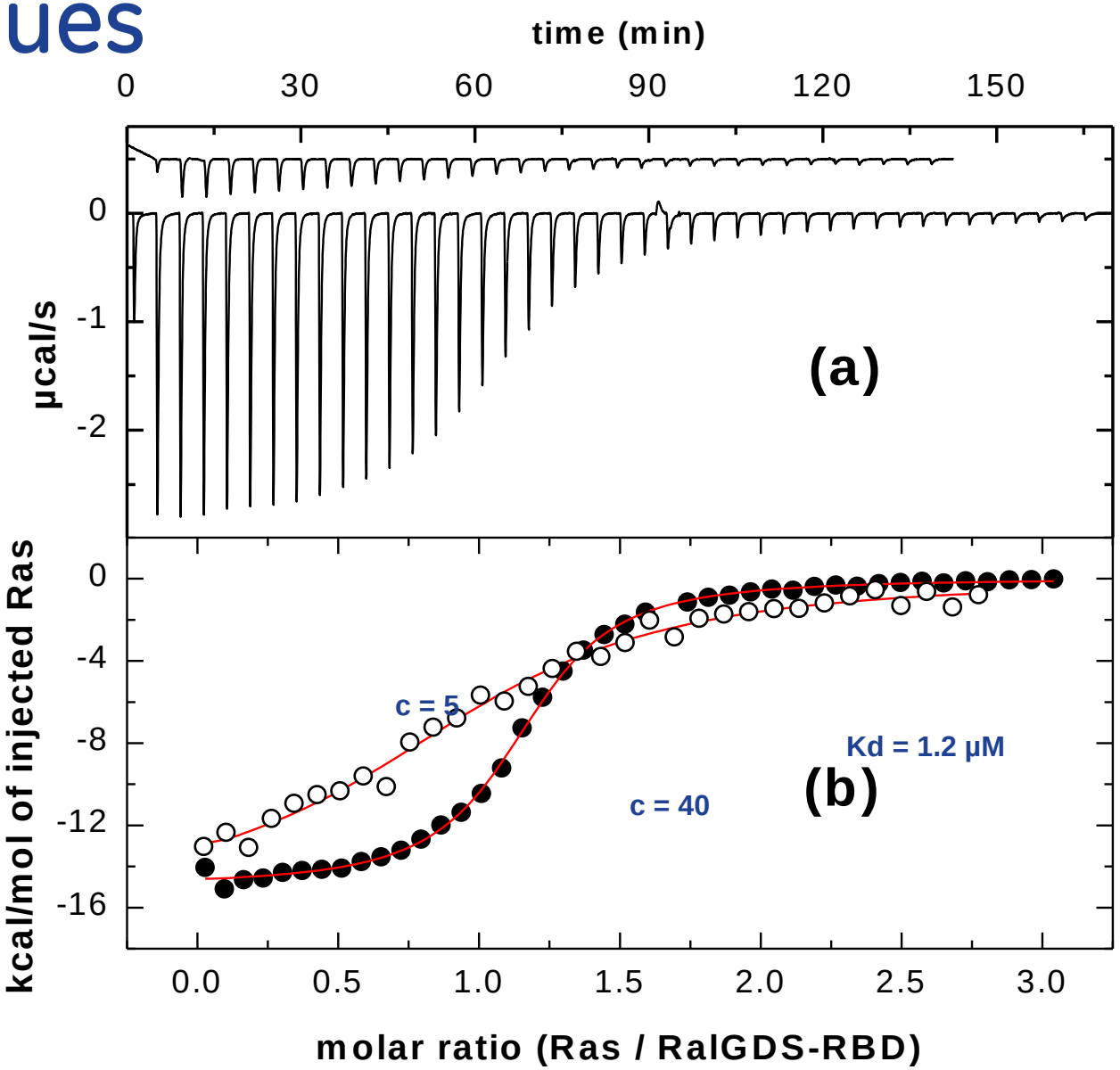
Kd = 100nM

[M] = 100nM, C=1

[M] = 5uM, C=50

[M]:[L] – 1:10 for n=1

# C Values


time (min)
0
30
60
90
120
150
0
-1
-2
µcal/s
(a)
0
-4
-8
-12
-16
kcal/mol of injected Ras
c = 5
c = 40
Kd = 1.2 µM
(b)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
molar ratio (Ras / RalGDS-RBD)

# C Values in ITC

$C = \{[M]_{tot} / K_D\} * N$

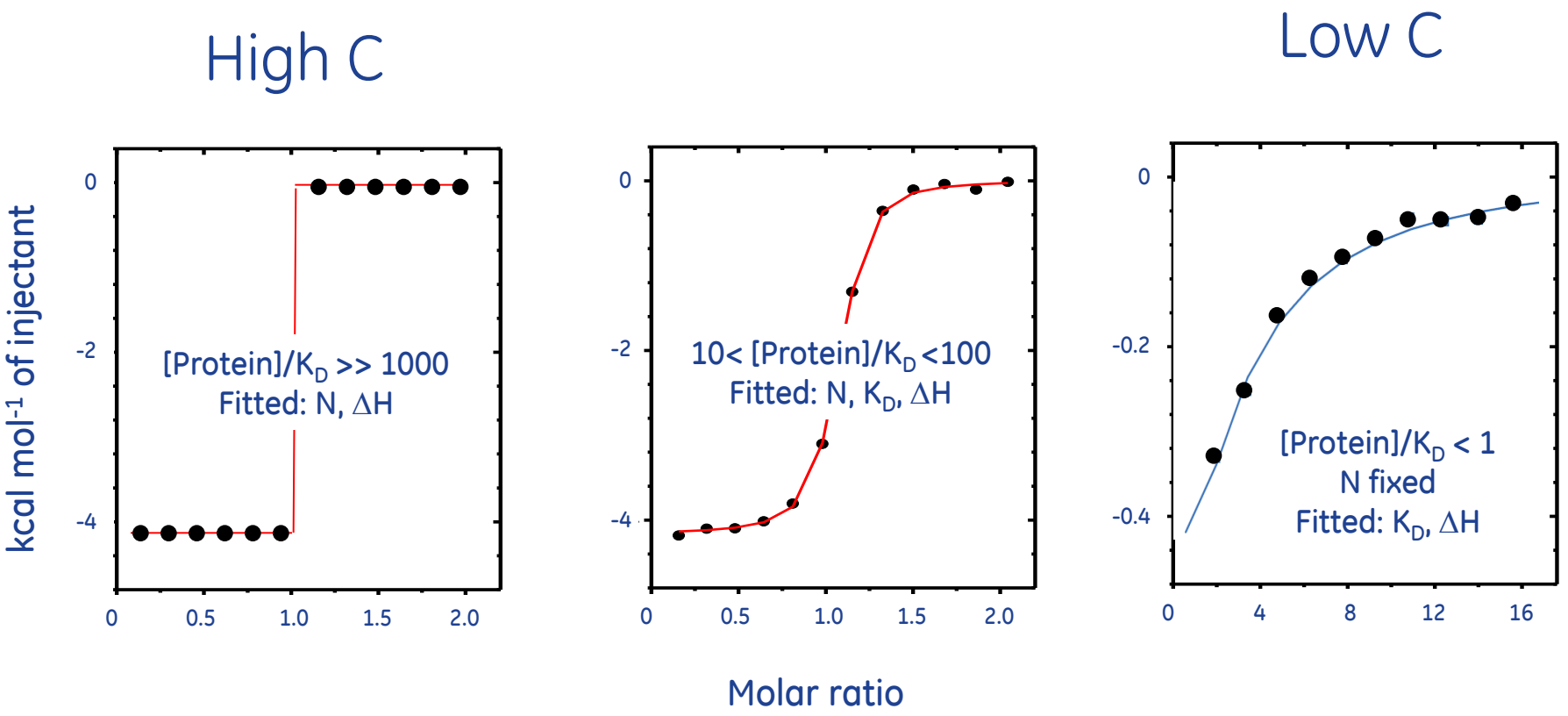
C = 20-100 very good

C = 10-500 good

C = 1-5 and 500-1000 OK

C = < 1 and > 1000 not wanted

# ITC experimental design



# ITC experimental design

| $K_D$ (Biacore) µM | [Protein] µM | [Compound] µM | [Protein] / $K_D$ |
|---|---|---|---|
| <0.5 | 10 | 100 | >20 |
| 0.5-2 | 30 | 300 | 15-60 |
| 2-10 | 50 | 500 | 5-25 |
| 10-100 | 30 | 40*$K_D$ | 0.3-3 |
| >100 | 30 | 20*$K_D$ | <0.3 |

Fixed stoichiometry

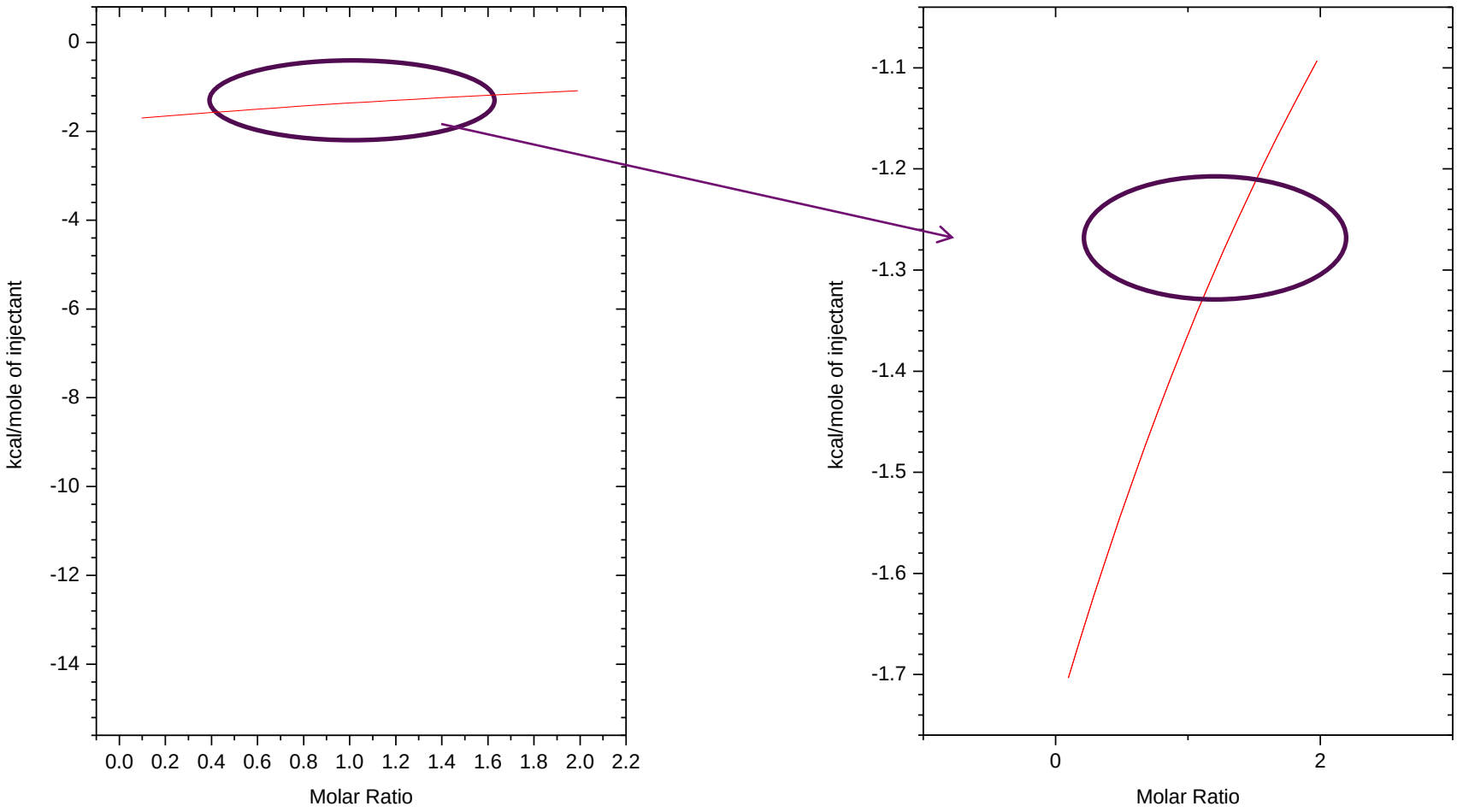
# Low C Experiments

Extending the applications of ITC

- E.g. Fragment Based Drug Discovery

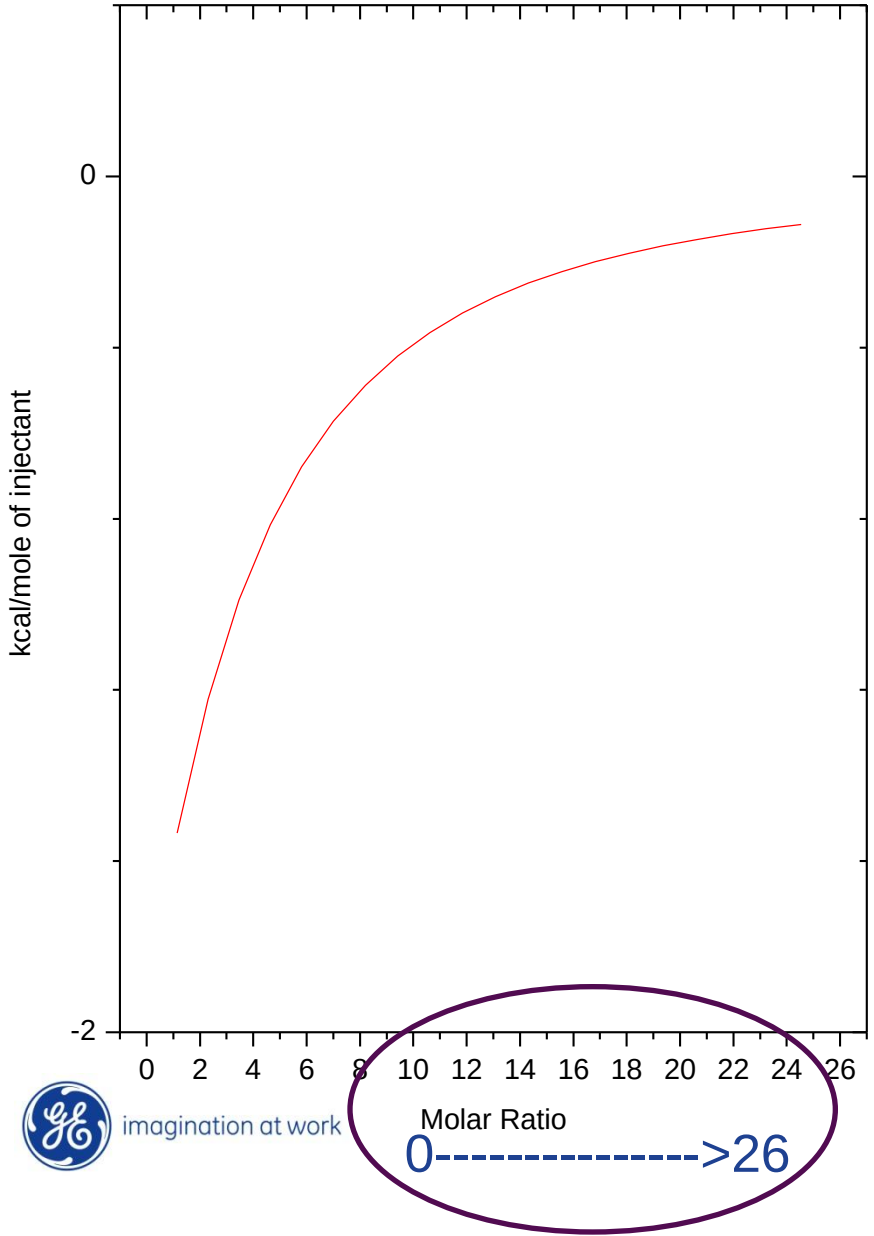
Saving Protein

# C Values-Low




Note different scales

# C Values-Low




Increase ligand concentration but not the ‘valuable’ protein

imagination at work

# Competition Experiments

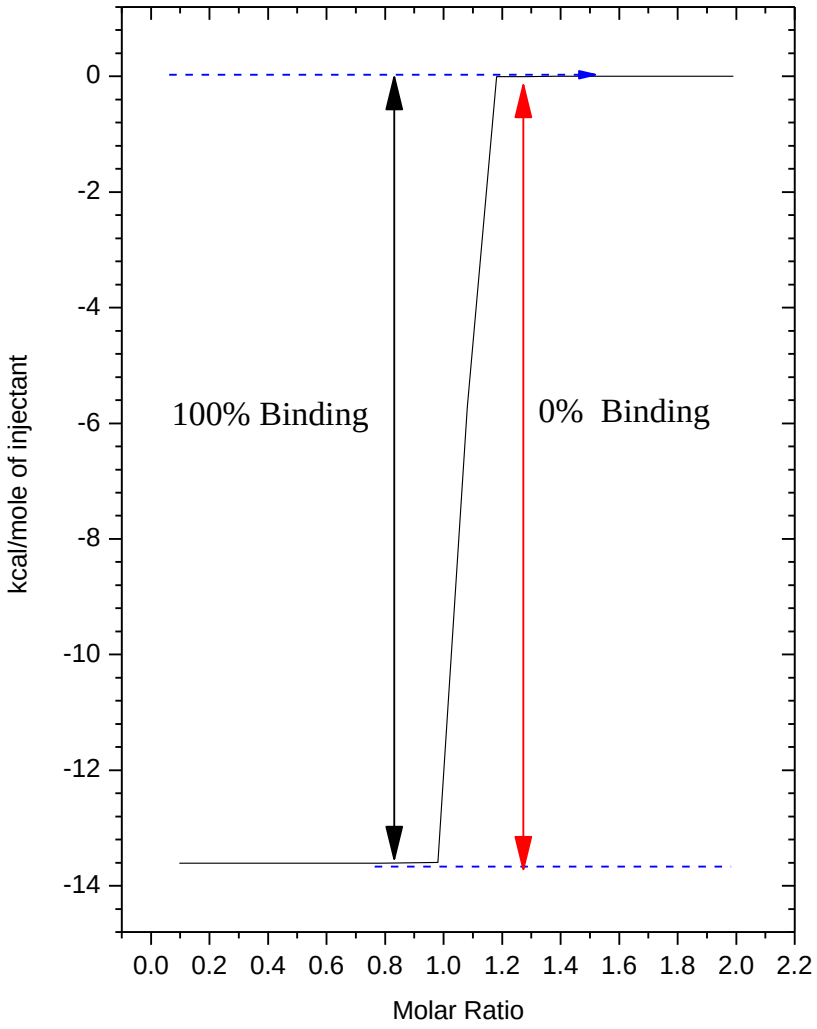
Extend the affinity range that ITC can be used

- Submillimolar ($10^{-2}$) to picomolar ($10^{-12}$)

# Competition Experiments

## High C Experiment

## Poor affinity estimates



# Competitive (displacement) ITC

Used to extend range of $K_B$ determined by ITC

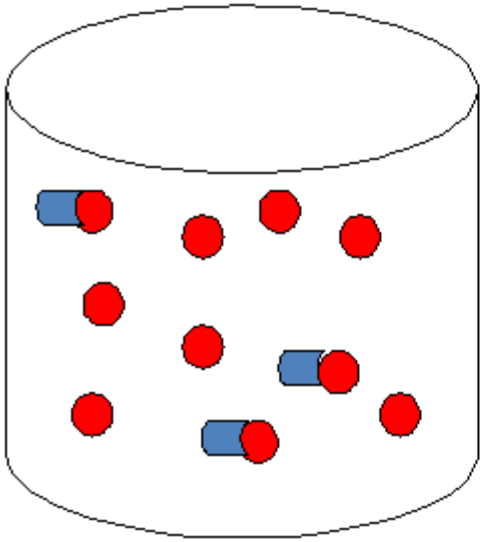
Tight binding ligand A and weak binding ligand B bind to same site on macromolecule

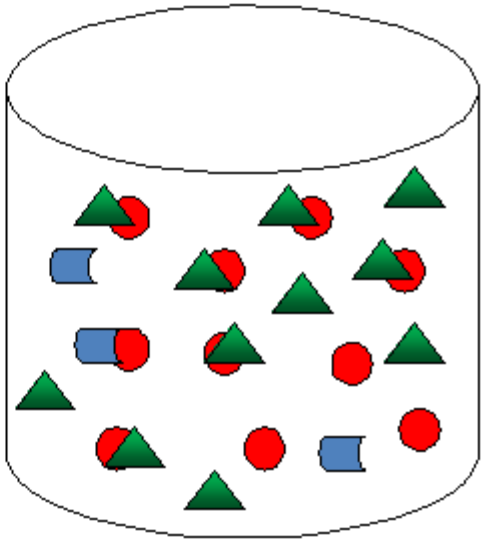
1st experiment: Ligand B titrated into macromolecule. Determine $K_B$ and $\Delta H$

2nd experiment: Macromolecule + ligand B in cell, titrated with Ligand A. Ligand A displaces ligand B.

Use displacement model for data analysis

# Tight Binders

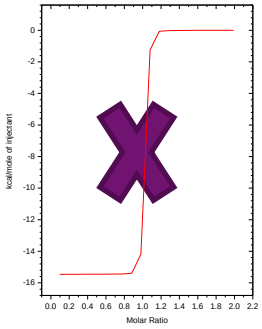


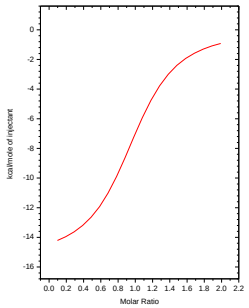


Protein

Tight Ligand

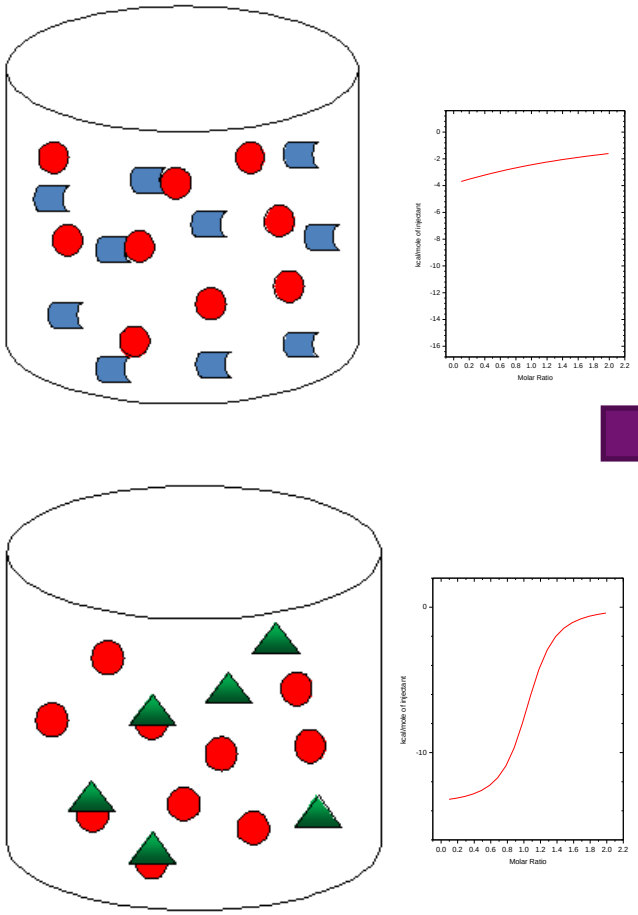
Competitor

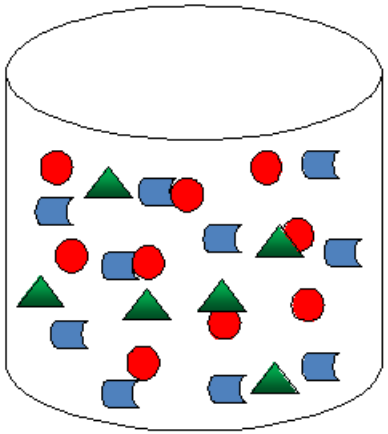


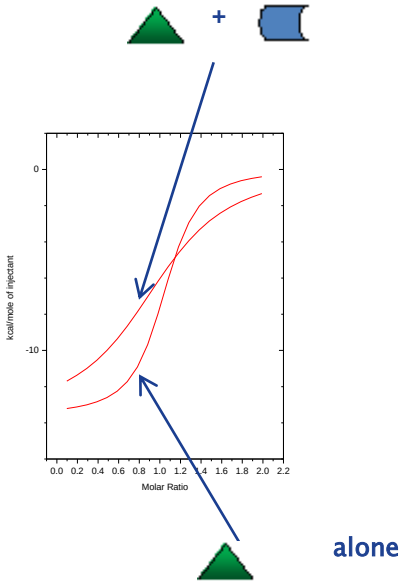




Software
can 'pull out' the $K_D$
of the tight one

# Weak Binders






Software can pull the Weak $K_D$ out

# Competition Experimental Design

$C = [cell]/K_{D,app}$

$K_{D,app} = (1/K_{D,S})/(1+1/K_{D,W}[W])$

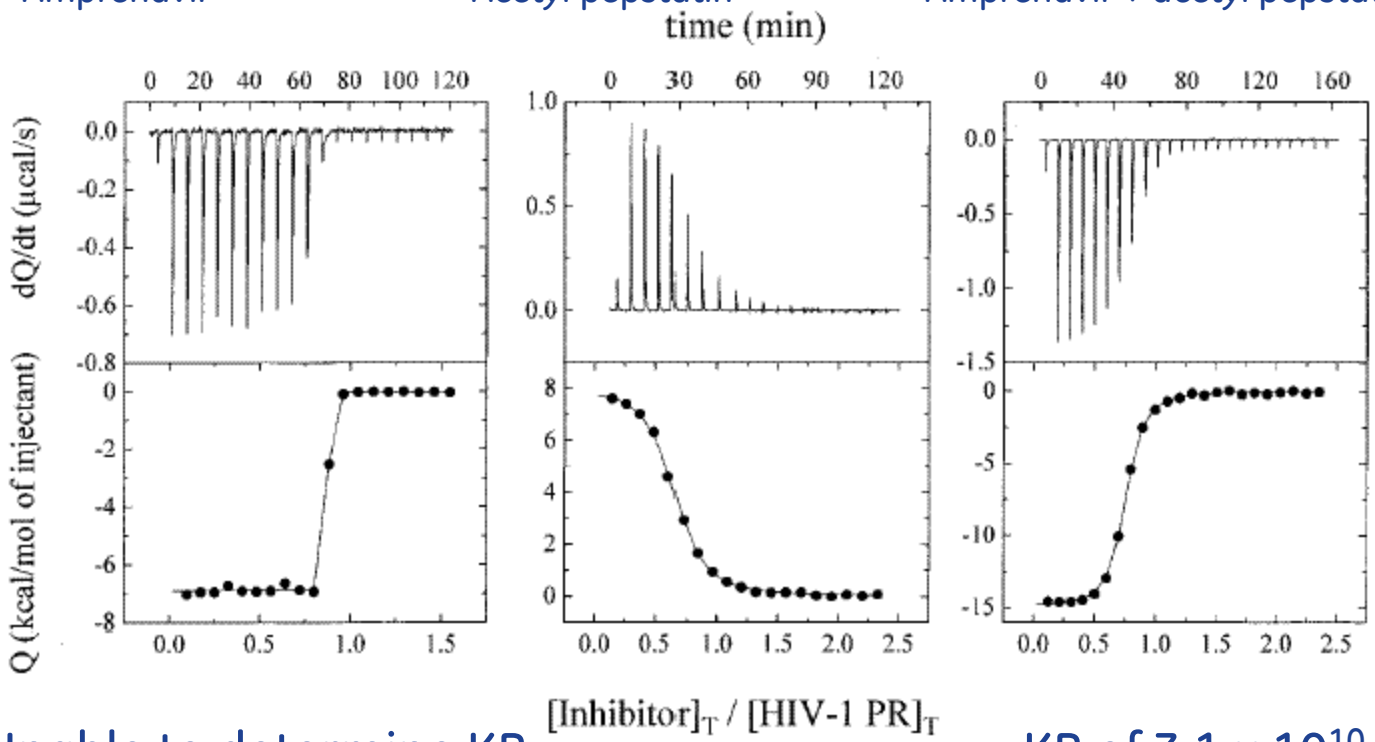
Where $K_{D,S}$ and $K_{D,W}$ are the affinity of the strong and weak binders respectively and W is the concentration of the weak binder

# Displacement ITC – HIV-1 Protease-Inhibitor Binding

Amprenavir

Acetyl pepstatin

Amprenavir + acetyl pepstatin



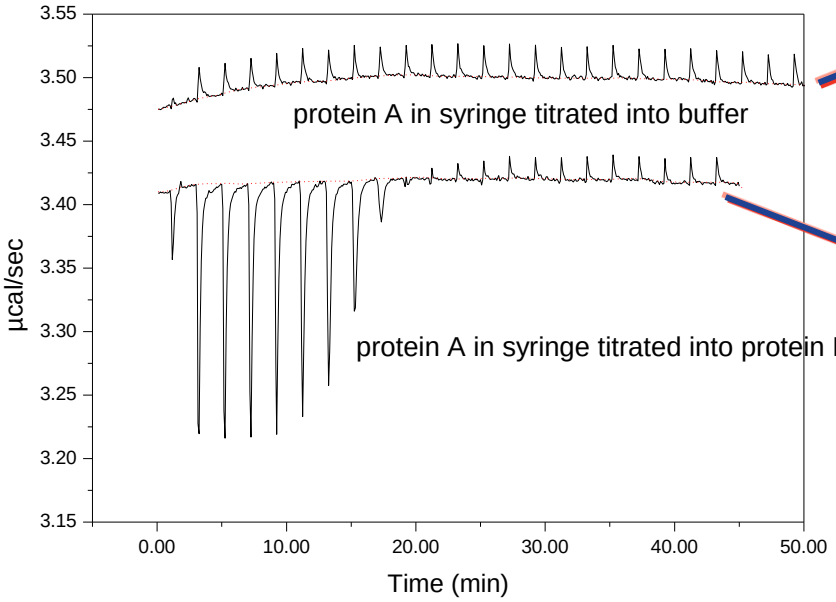

Unable to determine KB

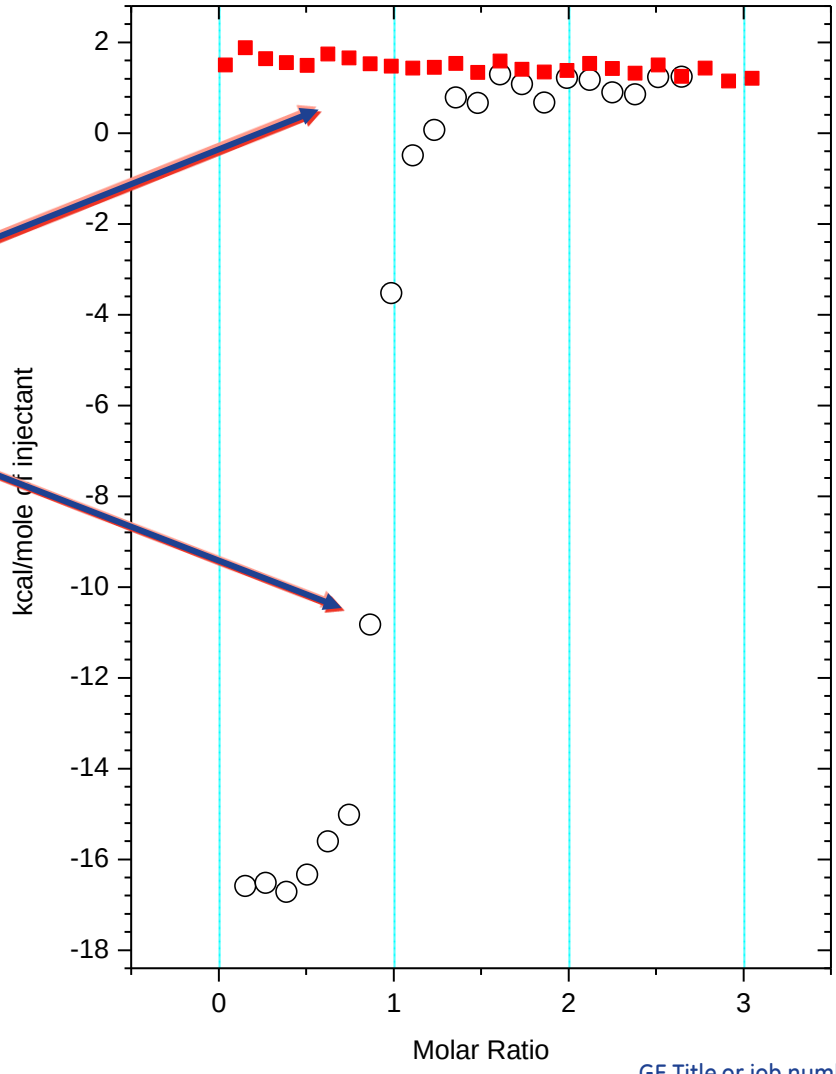
KB of 3.1 x $10^{10}$ $M^{-1}$

*Ohtaka, et al, Protein Sci. 11, 1908-1916 (2002)*

# ITC practical considerations

# ITC practical considerations



# Good Experimental Design

## Use the correct ‘C’ value

$$C = [M]_{tot}.n / K_D$$

C = 20-100 very good

C = 10-500 good

C = 1-5 and 500-1000 OK

C < 1 and > 1000 not wanted

# Ligand concentration

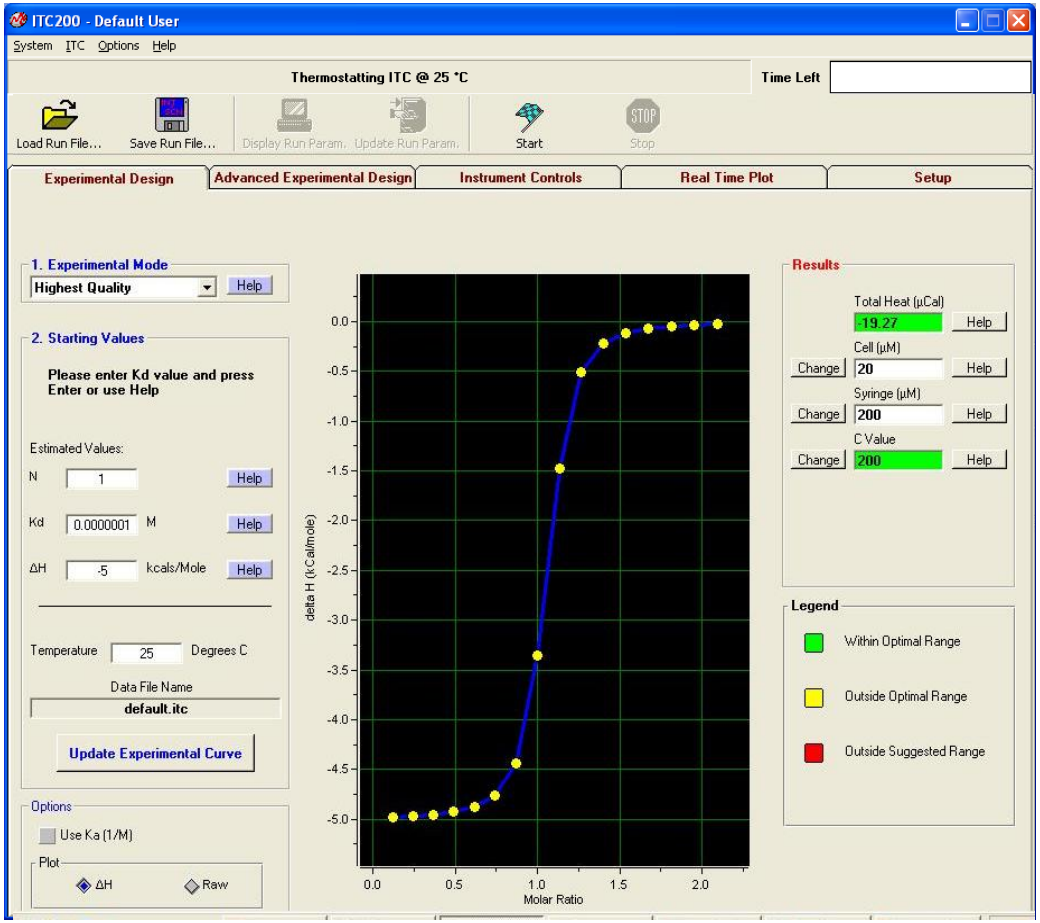
[L] = 10 to 20 x [M]

[L] - Minimum 50 μM

May need to be adjusted based on experiment

At end of ITC, for N of 1, final [L]/[M] ratio should be 2 to 4 to ensure saturation of all M binding sites

# $iTC_{200}$ Experimental Design Tab



# With little prior knowledge

## Good Starting Conditions

100μM Ligand in the Syringe and

10μM Macromolecule in the cell

16 x 2.5 μl injections

Detect $K_D$s of 10 μM to 10 nM

Ideal for $K_D$s of 2 μM 100 nM

# Sample preparation

Use dialysis or buffer exchange column

Check calibration pipettes-by weight

Retain the dialysate/exchanged buffer

Adopt and stick to a reproducible protocol

# ITC – Choice of Buffer

What buffer(s) are protein and ligand stable in? What pH?

Buffers used for other binding studies

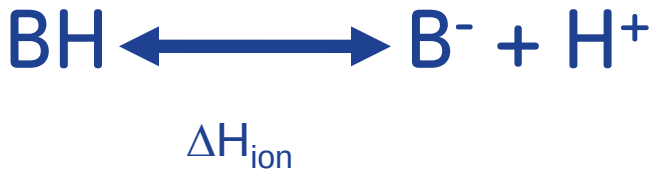
Solubility

Requirements of additives for binding, solubility or stability

- Salt
- Detergent
- Reducing agent
- DMSO
- Other

# Choice of buffer

Buffers have ionization enthalpies:




Use buffers with $\Delta H_{ion} \sim 0$

Including; phosphate, acetate, formate, citrate, sulfate, cacodylate, glycine

Quaternary amines (e.g. Tris) have high $\Delta H_{ion}$

# Choice of buffer

Avoid DTT

- Unstable and undergoes oxidation
- High background heat

Use b-mercaptoethanol & TCEP

TCEP is not stable in phosphate buffer

Use conditions in which your protein is ‘happy’

# Sample preparation: small molecule ligand

If ligand is too small to dialyze, be sure material is desalted prior to final preparation

Use final dialysis buffer of macromolecule to dissolve ligand

Match pH

# ITC – “Reverse Titration”

Can also have ligand in cell – lower concentration requirement.

Have macromolecule in syringe at appropriate (higher) concentration, Need to be sure can have protein at the appropriate concentration.

At saturation: no more free ligand in ITC cell

If other than 1:1 binding, use “ligand in cell” option for curve fitting. Different model used.

# $iTC_{200}$ Experimental design

Injection volume and duration

- 0.5 to 3 μl ((range 0.1-38 μl)
- Injection rate is 0.5 μl/sec
- Spacing
- Typical 120 secs-may want to extend to 180 seconds or more if using no feedback with large heats
- Be sure baseline returns before next injection
- If this does not occur, increase time between injections

Filter period

- 5 sec or less recommended
- Can be increased for slow reactions

# $iTC_{200}$ Experimental design

Typical temperature: 25 °C

Number of injections: 12-18

Reference power

- Instrument baseline
- Set 5 μcal/sec
- If very exothermic, increase setting so ΔP does not go below zero

Initial delay

- 60 sec minimum
- Establish baseline before 1st injection

# $iTC_{200}$ Experimental design

Feedback mode

- High feedback for most ITC experiments
- Low or No feedback will give better S/N but will take longer time (increase time between injections) – normally used when working with small heats

Stirring speed

- 1000 rpm (1500 for SIM)

# Auto- $iTC_{200}$

Macromolecule/protein (for cell):

Need 400 μl in 96 well plate

Ligand (for syringe):

Need 120 μl in 96 well plate

Set appropriate scripts for cell/syringe filling and cleaning

# ITC – Enthalpy Changes

$\Delta H_{observed}$ by ITC is total of :

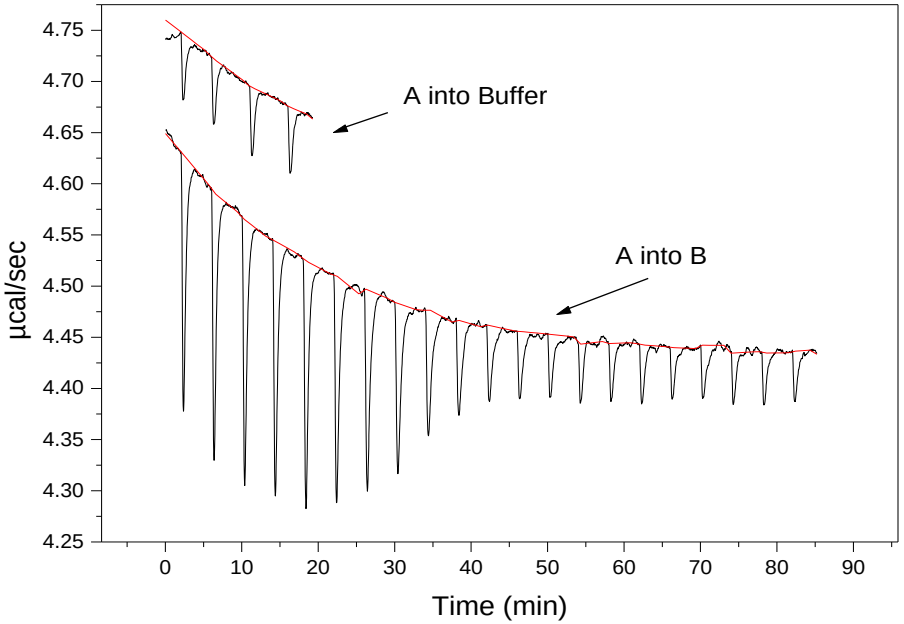
- $\Delta H_{binding}$
- $\Delta H_{ionization}$
- $\Delta H_{conformation}$

Any non-specific effects (buffer mismatch, pH mismatch, heat of dilution, heat of ligand dissociation)

***Need to account for these effects by appropriate controls and experimental conditions***

# Controls

## Injection of syringe material into buffer-




Peaks should be similar in magnitude to those at the end of the actual titration experiment and constant

# Controls

If they are constant-

subtract average peak size from the experimental data (Using the ‘Math’ command)

# Controls

If they are not constant

Buffer Mismatch –e.g. solvent component missing

Dissociation of the syringe material upon dilution into buffer- impact on apparent $K_D$

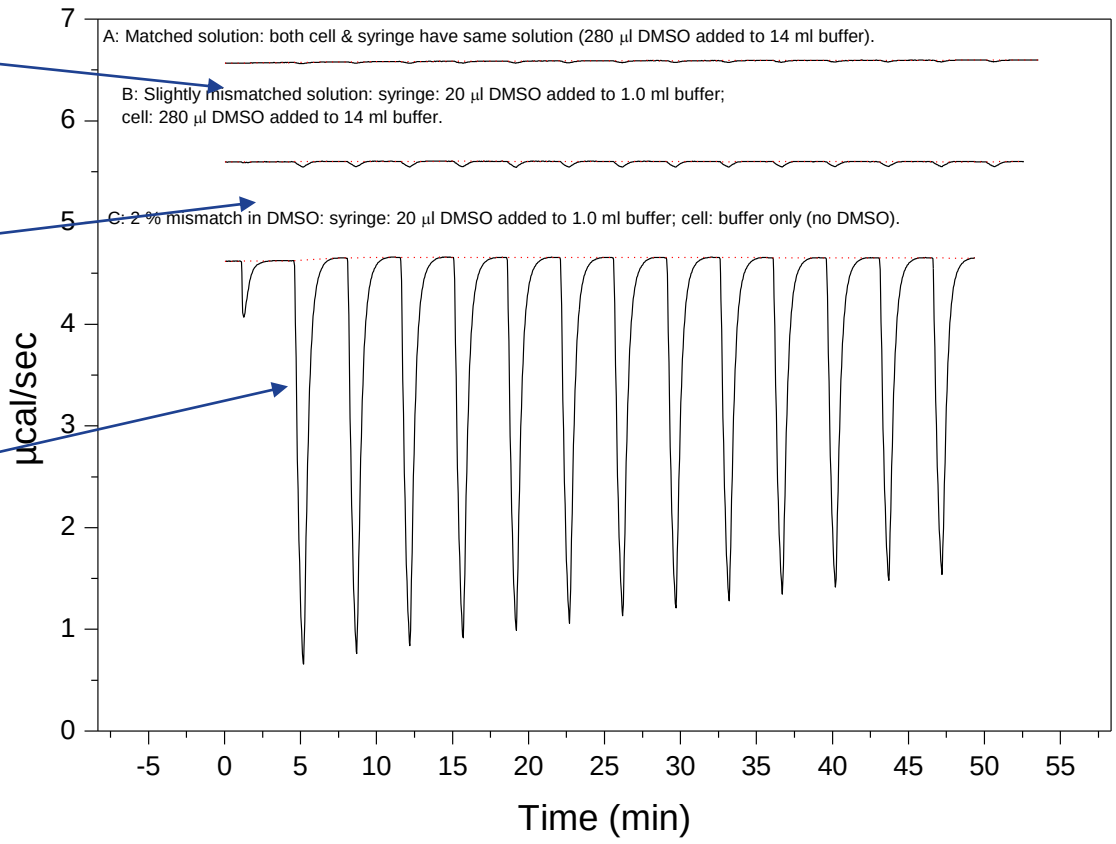
Genuine change in heat of dilution with concentration.

# Buffer Mismatch

2% DMSO into
2% DMSO

2% DMSO into
1.95% DMSO

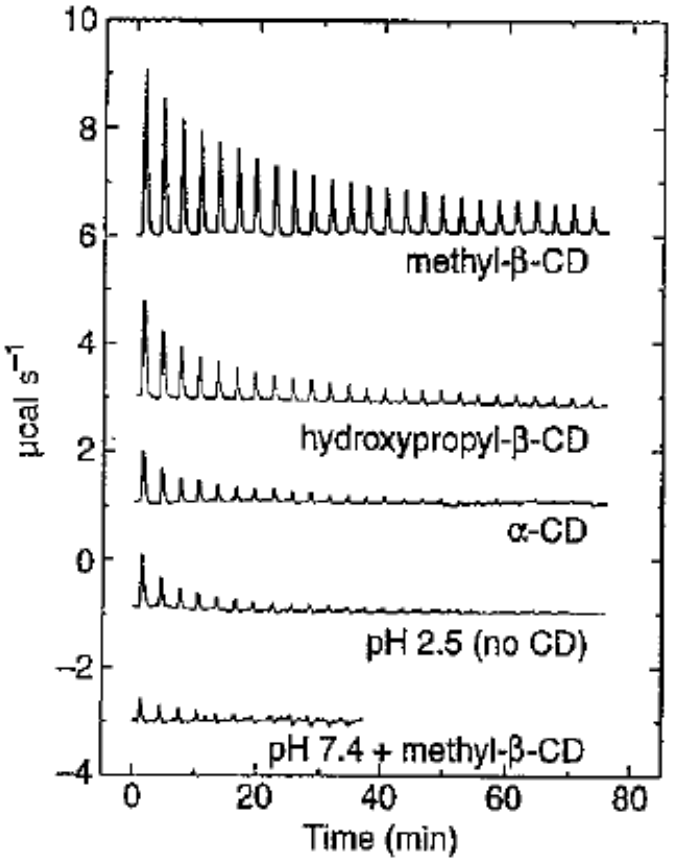
2% DMSO into
0% DMSO



# Dissociation of Syringe Material into Buffer

Calorimetric dilution data showing the effects of different ligands on dilution of insulin

Ref: Lovatt M, Cooper A and Camillerri P (1996) *Eur. Biophys. J.* **24**:354-357



# Changing Heats of Dilution

The control experiment should be fit to a straight line – (or geometric function (if required- Caution!)

This best fit line can then be subtracted from titration experiment before fitting to the appropriate model.

# For Each ITC Experiment

Start with clean cell and syringe

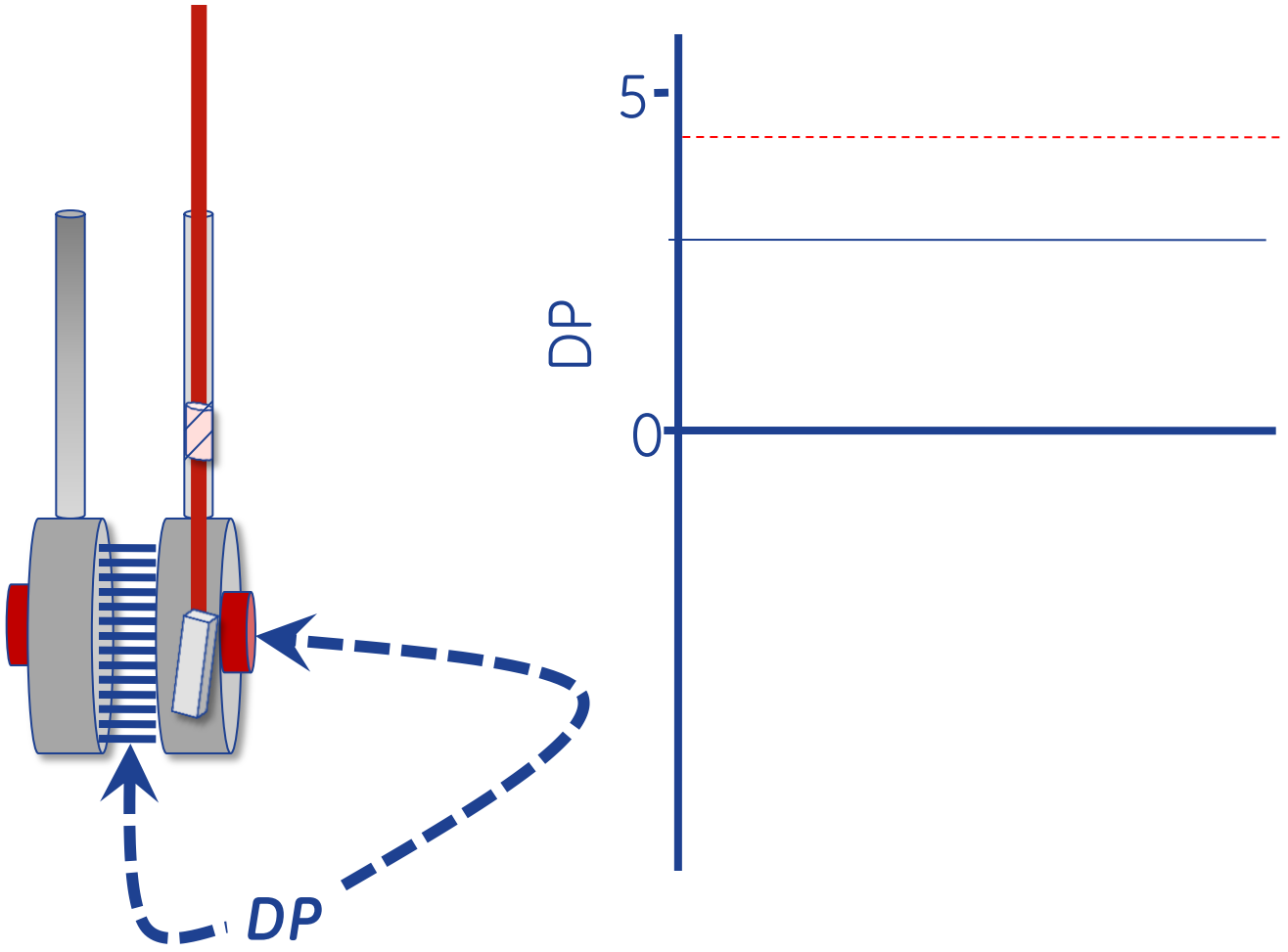
Prepare macromolecule and ligand in **matched buffer**

Perform control titration(s) to establish heat of dilution

Set appropriate scan parameters to generate full binding isotherm

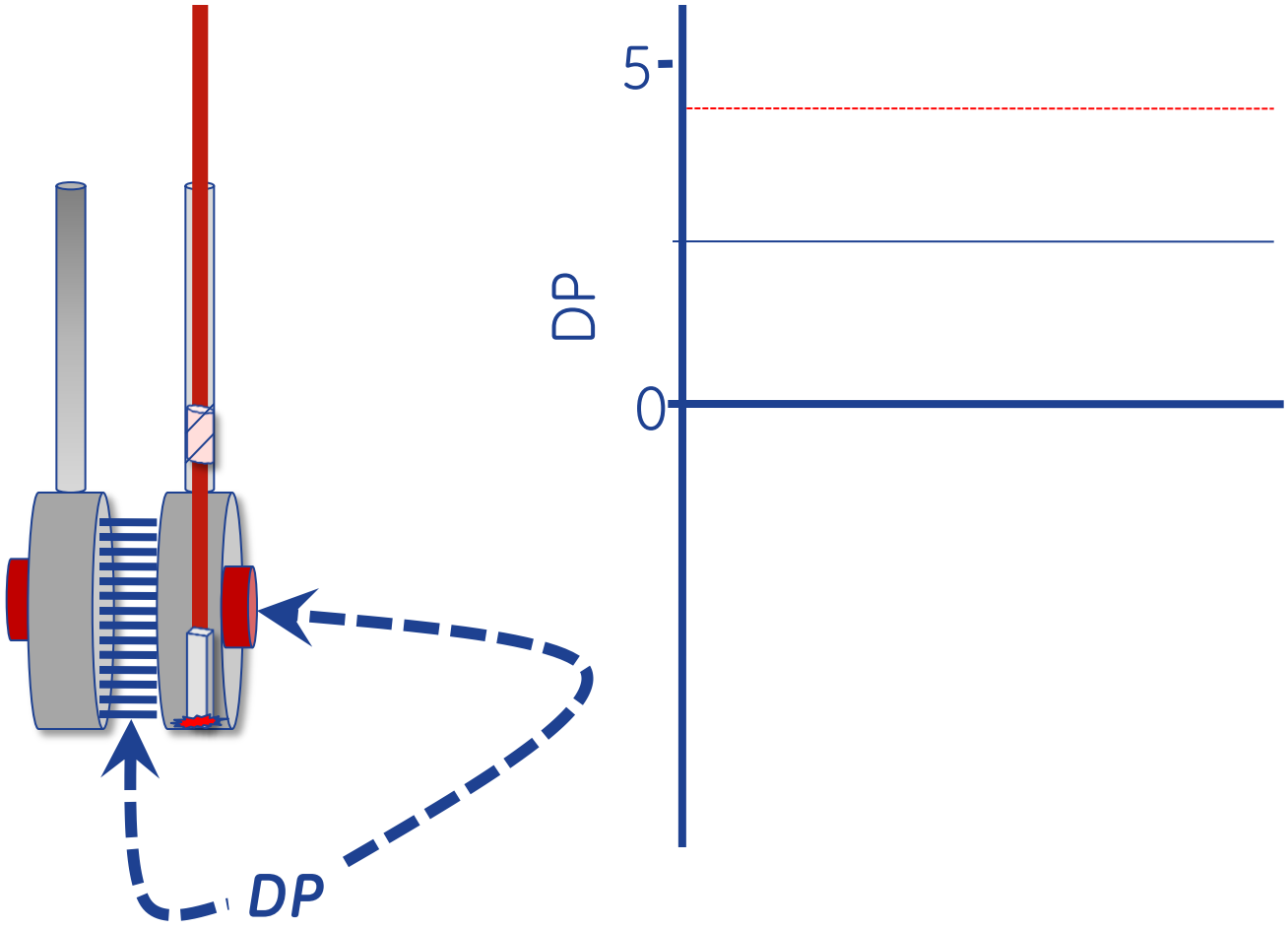
# Troubleshooting

# Bent Syringe



# Syringe Height

## $iTC_{200}$ - Syringe Holding Nut Loose

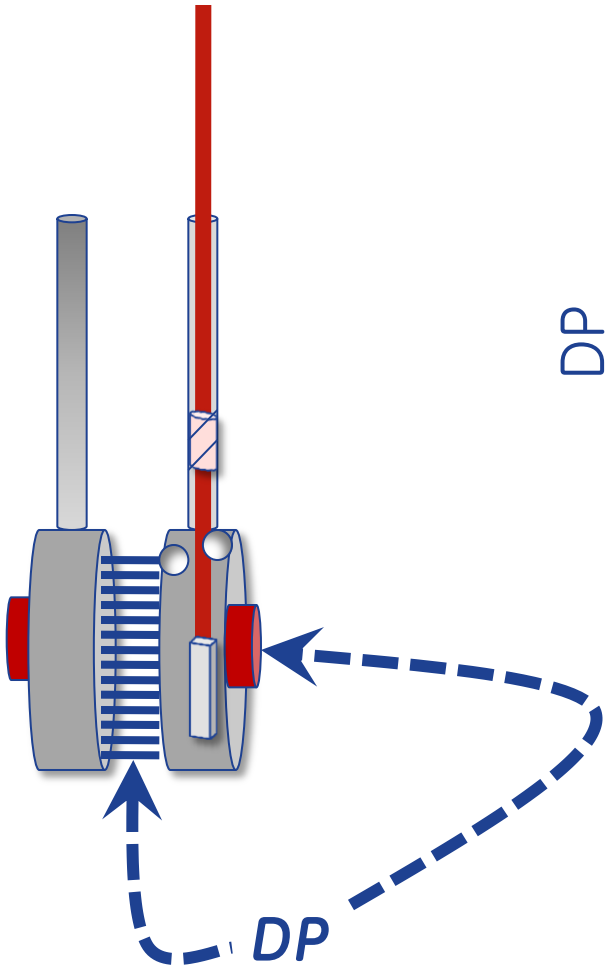


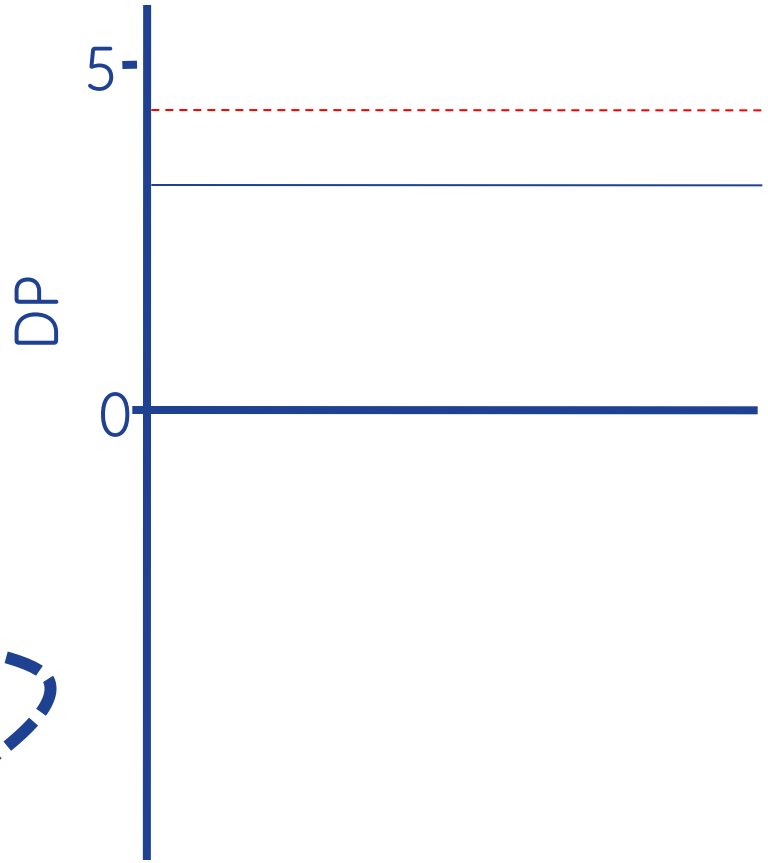
# Baseline Position/Drift

Baseline position is the first diagnostic for data quality-information on

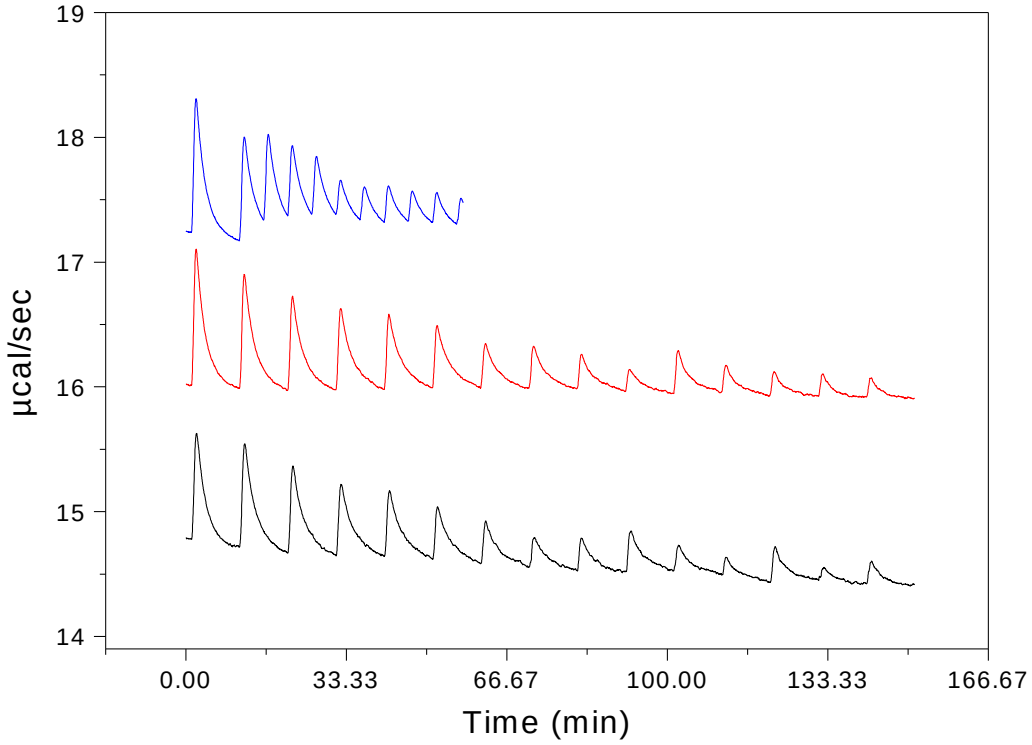
- Cell cleanliness
- ‘Sticky’ proteins
- Air Bubbles
- Time between injections

# Bubbles

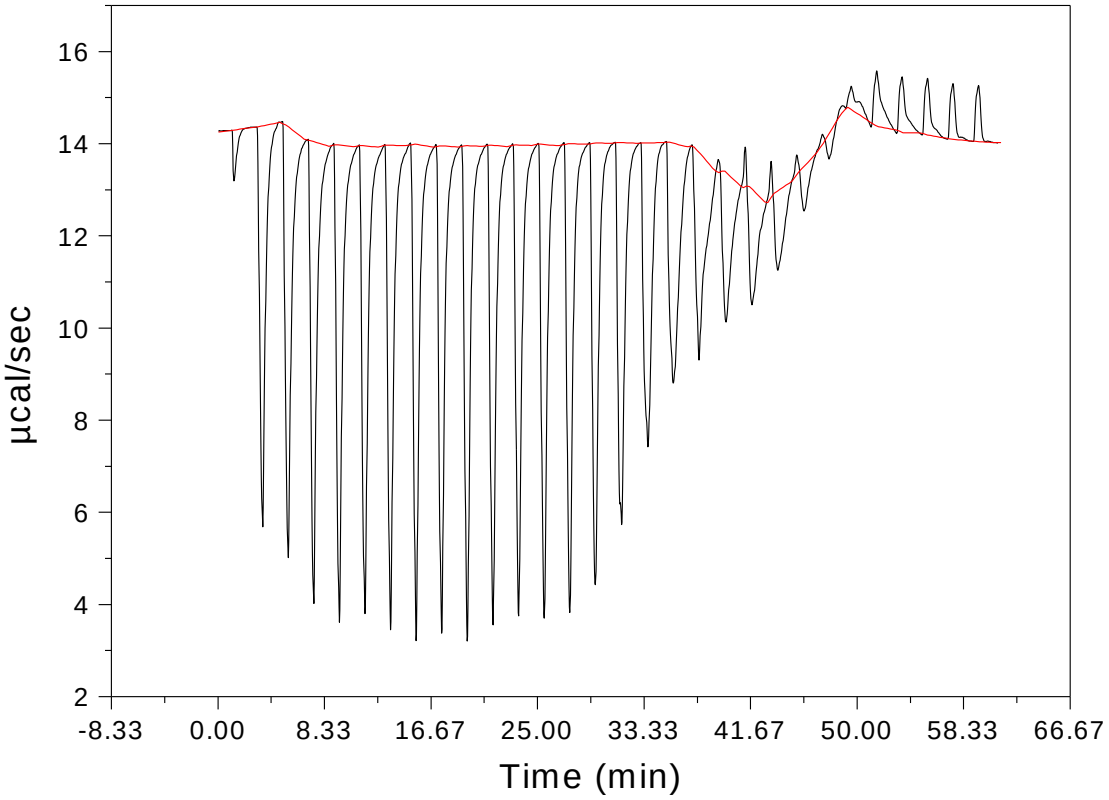


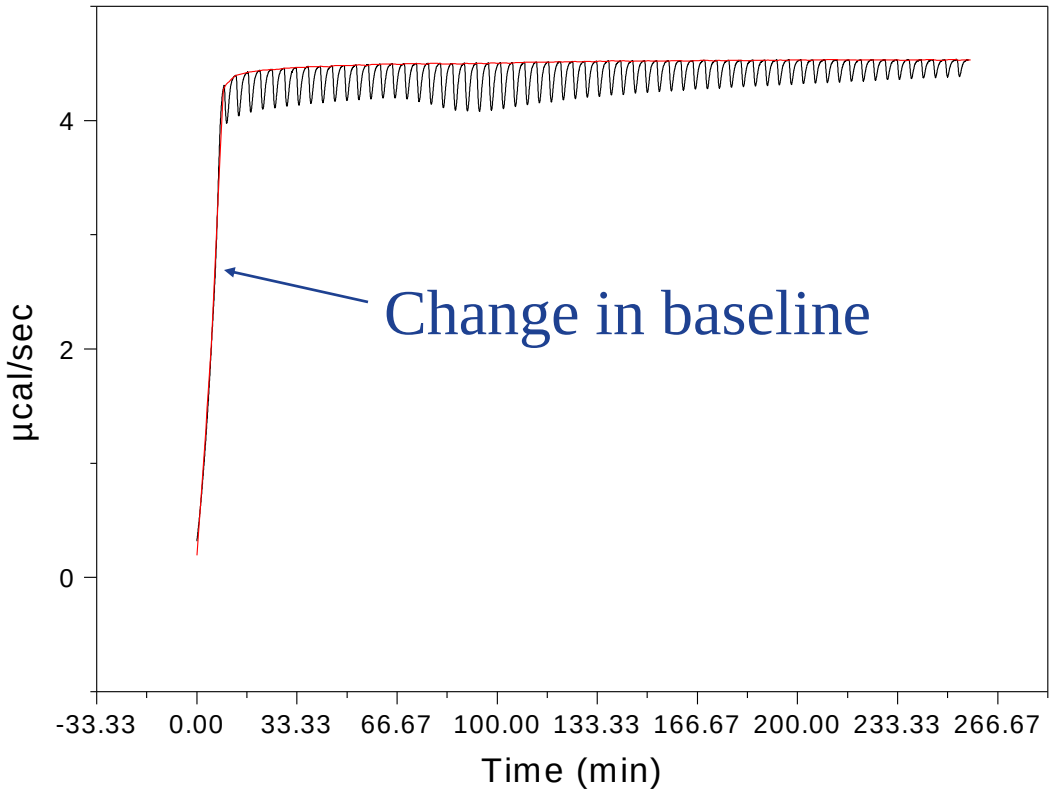
# Not long enough between injections



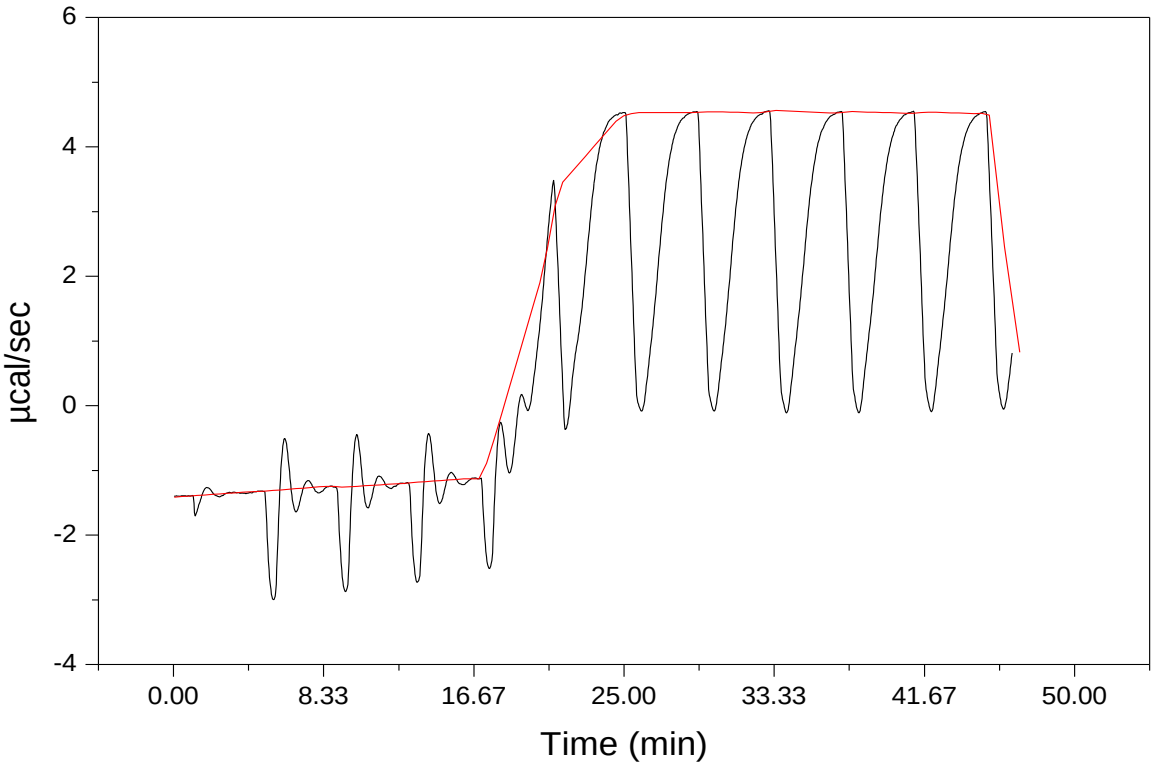
# Not long enough between injections



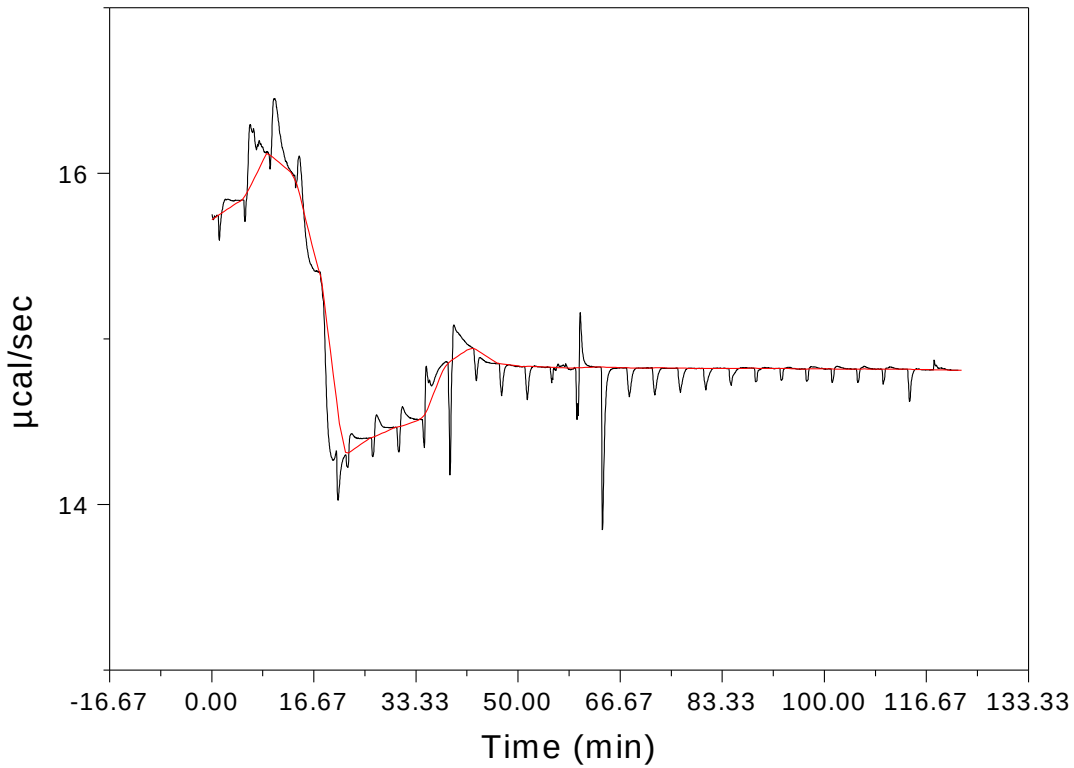
# ‘Sticky’ Proteins or Cleanliness



# ‘Sticky’ Proteins or Cleanliness



# ‘Sticky’ Proteins or Cleanliness



# Non sigmoidal binding isotherm

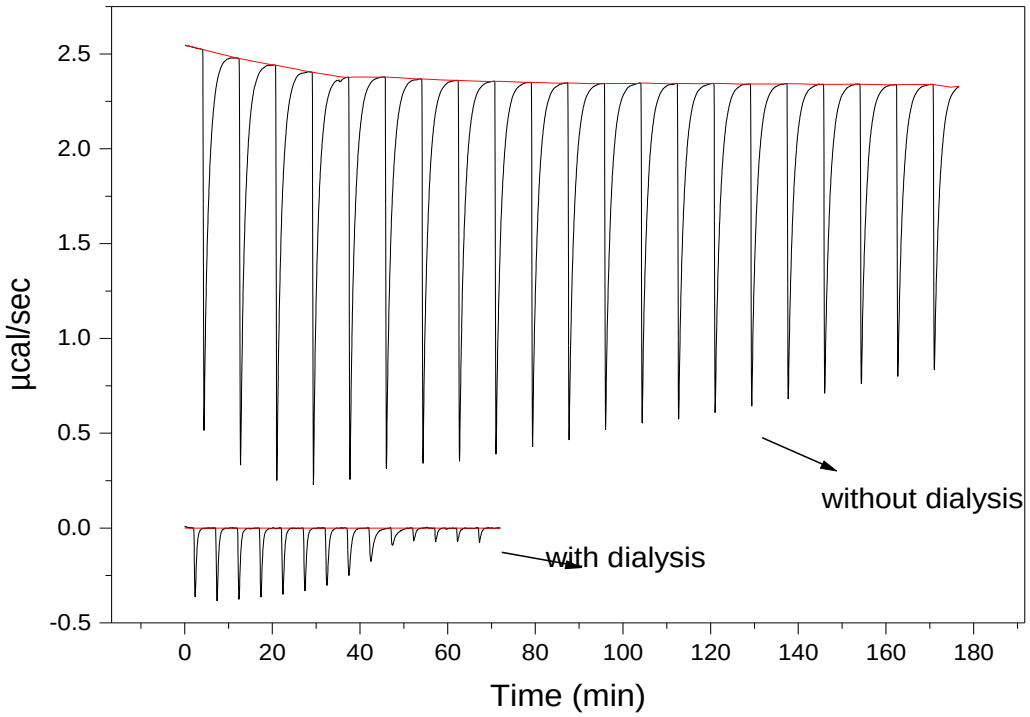
No Binding

No Heat

Buffer mismatch

More than one binding event

# Buffer Mismatch-No Dialysis

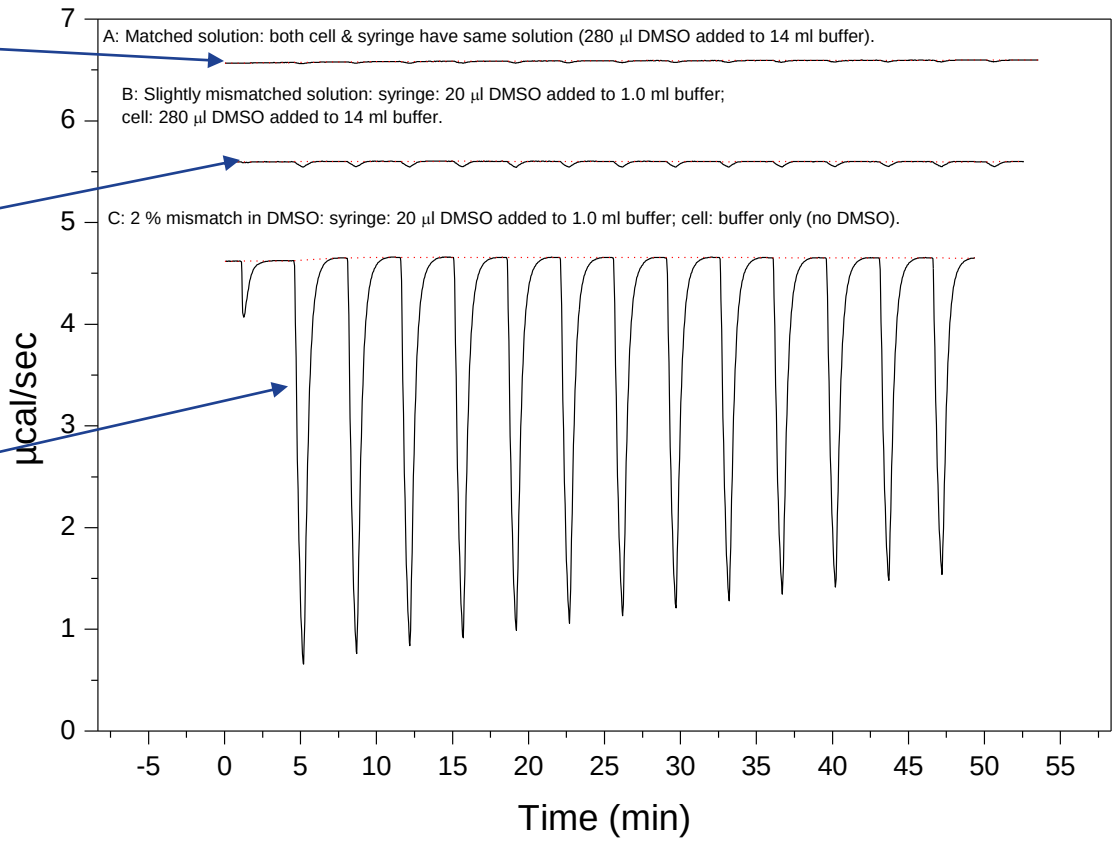


# Effect of DMSO Mismatch

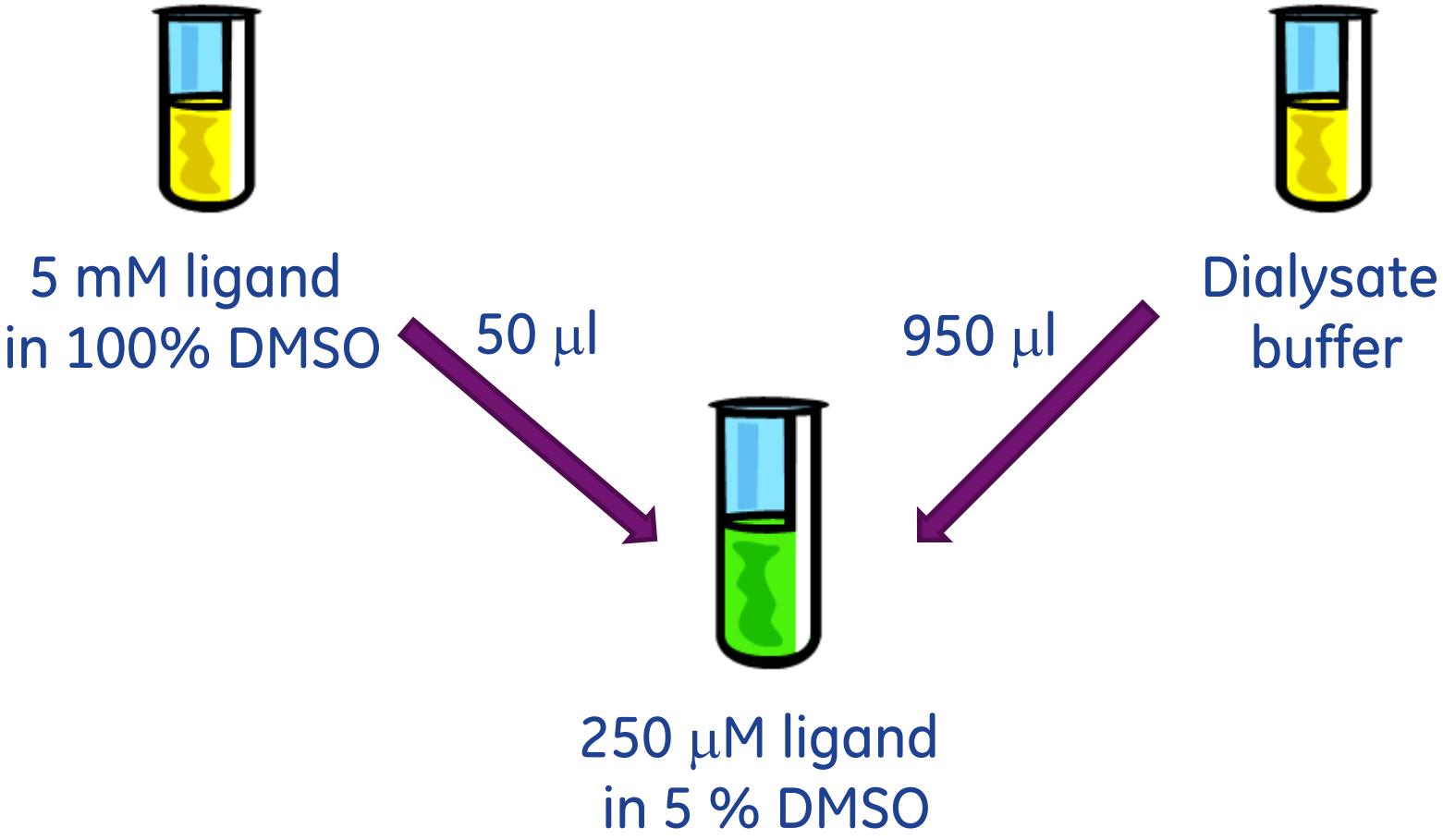
2% DMSO into
2% DMSO-same stock

2% DMSO into
2% DMSO-separate stocks

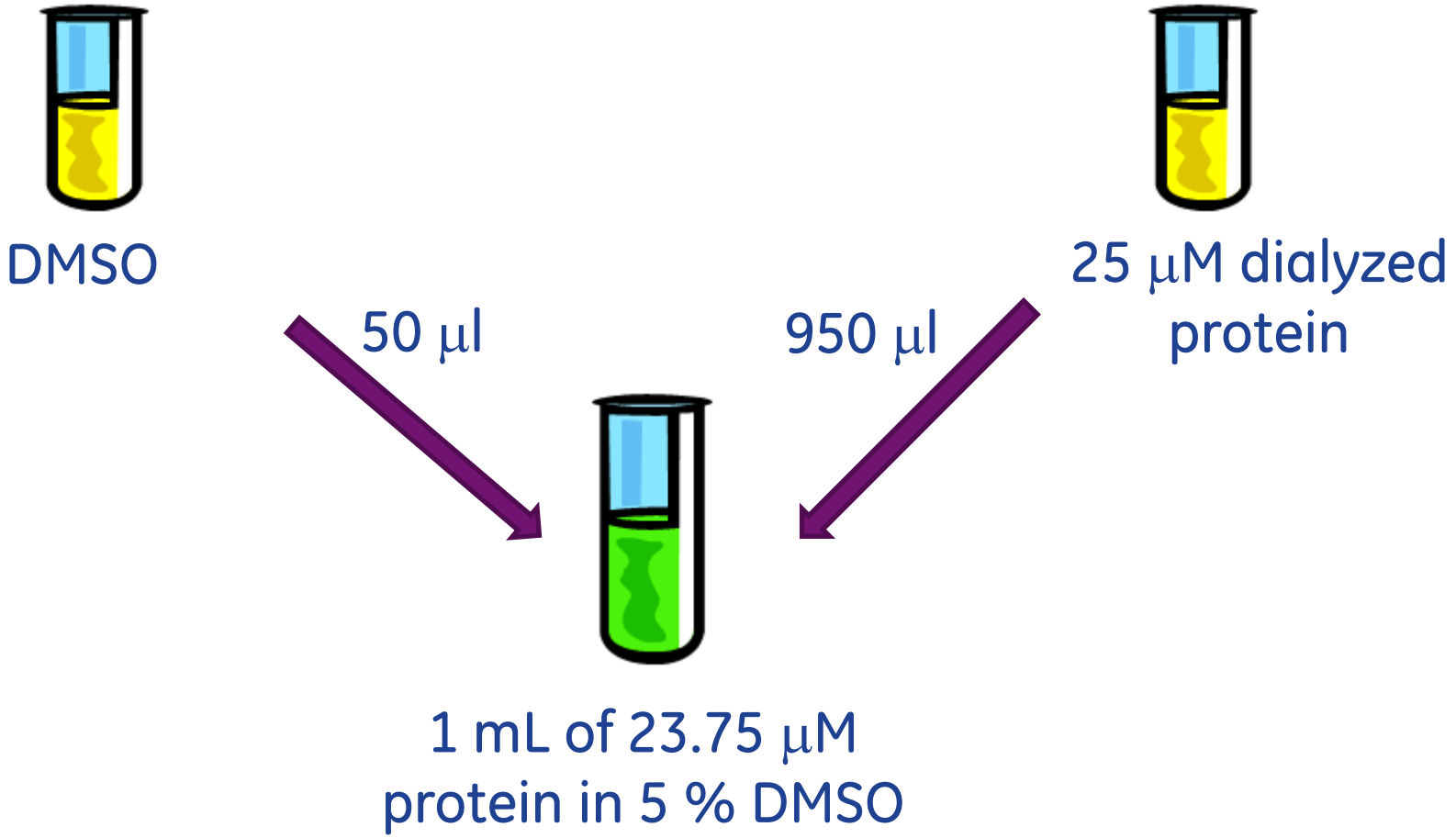
2% DMSO into
0% DMSO



# Ligand preparation from DMSO



# Match DMSO in the protein solution



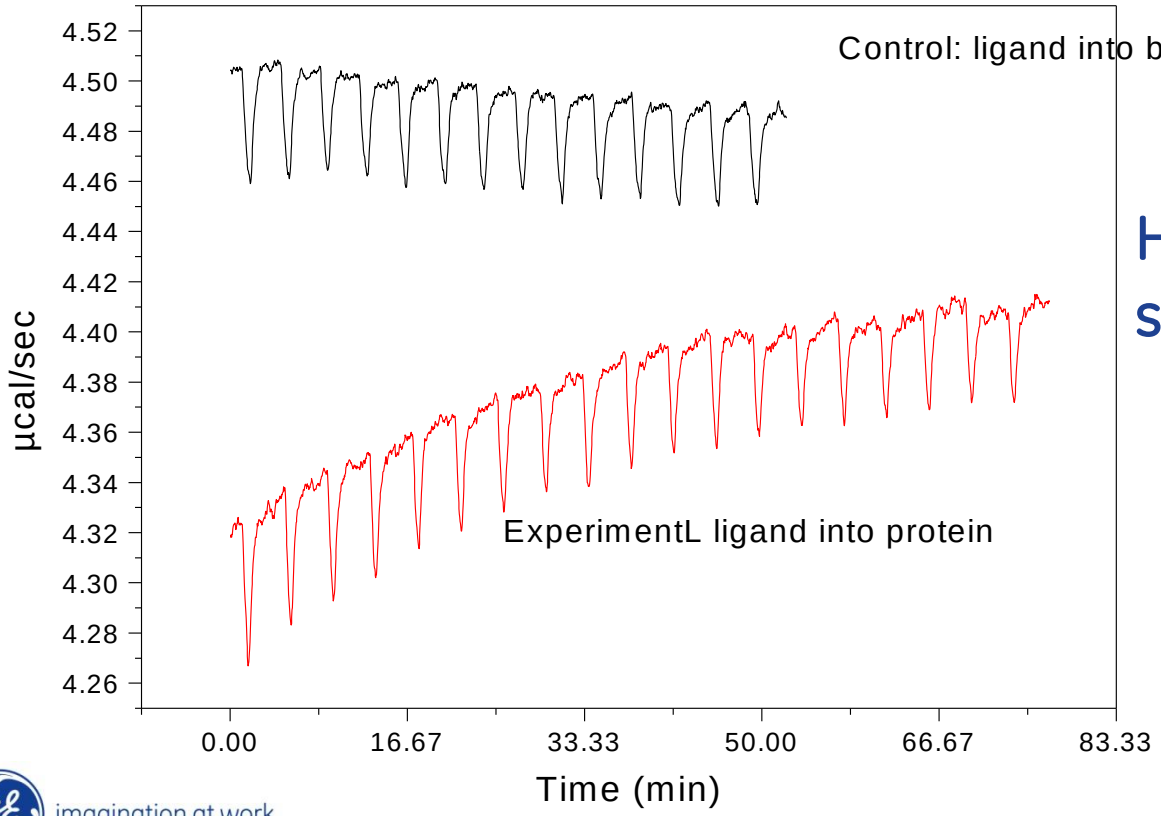
# The Cure

Dialyze or use desalting column

Check for additives that are not in both cell and syringe- Also-ask what was sample purified from e.g. was protein lyophilized in buffer and not dialyzed

Check pH of final solutions-should differ by less than 0.1 pH units. This issue is common when working with high concentrations of ligand-e.g. 500 μM and above-weak binding

# ITC: low heat




Heats for experiment same as control

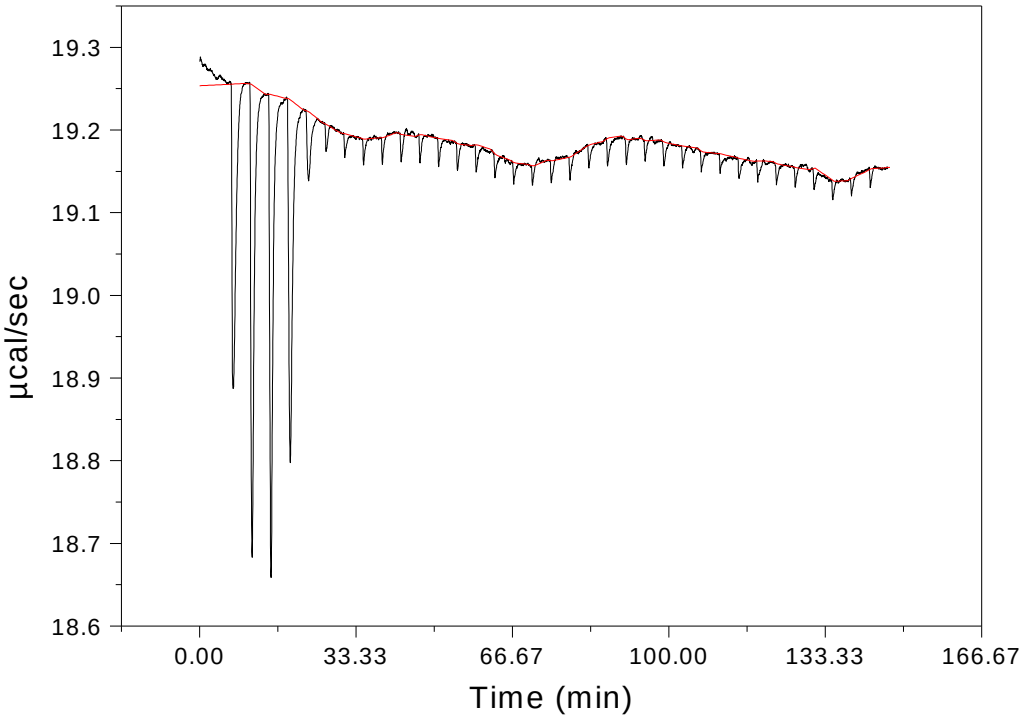
# The Cure

Change experimental temperature by at least 10 °C

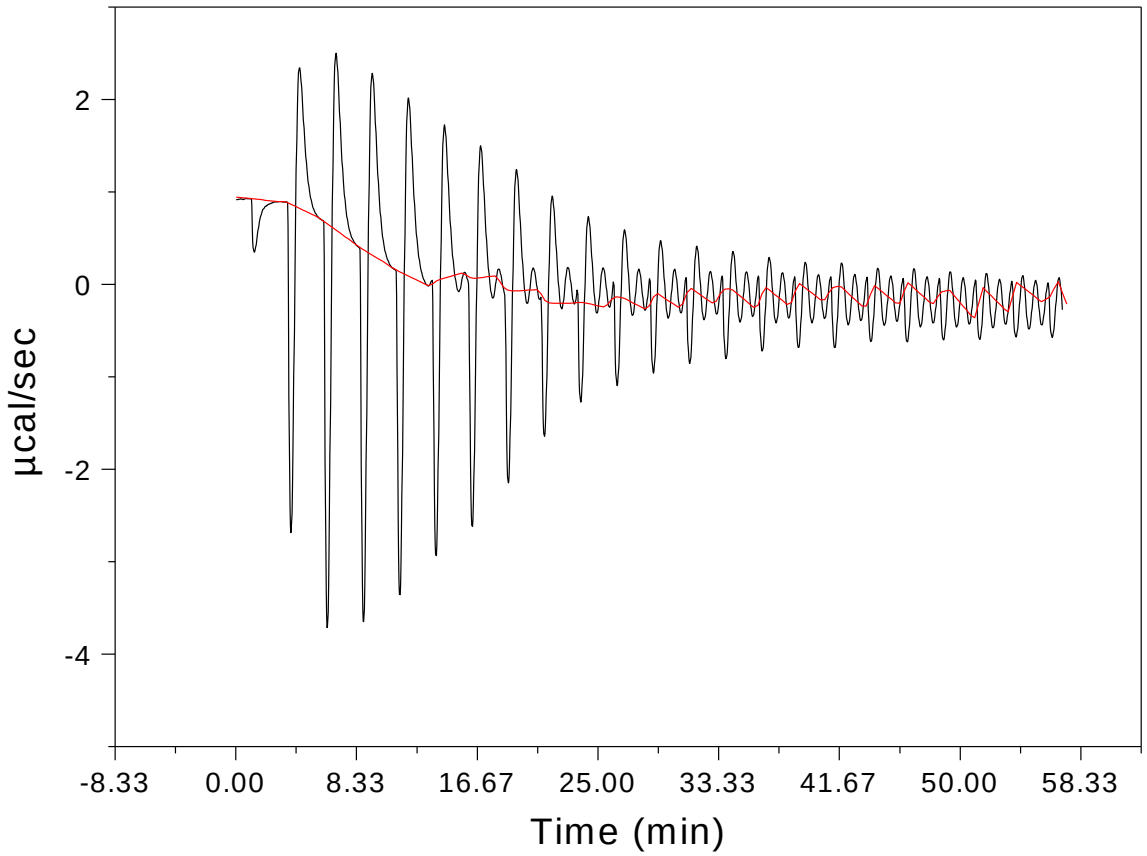
AND/OR

Increase sample concentration

# Unexpected Stoichiometry



# Instrument reference power too low



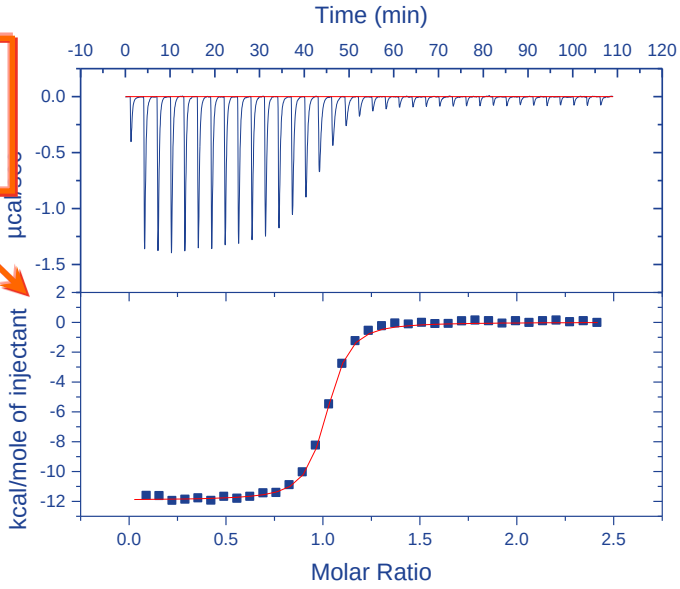

Oscillating signal:
Power below 0

# Insoluble Ligands

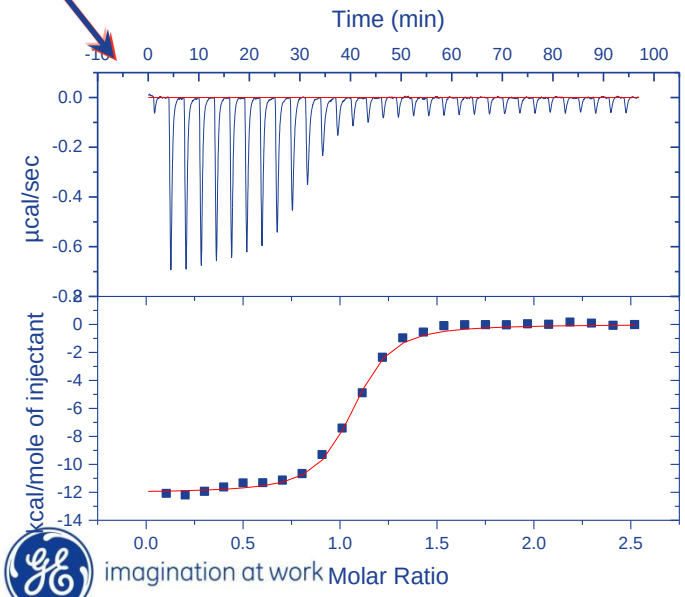
‘One site’ interactions are symmetrical and as such the ligand can be put in the cell and the protein in the syringe

# Cure-Reverse the Titration

29.5µM Protein titrated with
1.1mM Compound




11.5µM Compound titrated with
179µM Protein




| *Parameter* | *Ligand in syringe* | *Ligand in cell* |
|---|---|---|
| n | 0.99 | 0.97 |
| $K_d$ | 104 nM | 105 nM |
| $\Delta G^o$ | -9.4 kcal/mol | -9.4 kcal/mol |
| $\Delta H^o$ | -11.9 kcal/mol | -12.4 kcal/mol |
| $T\Delta S^o$ | -2.5 kcal/mol | -3.0 kcal/mol |

# Stoichiometry

"N" is the average number of binding sites per mole of protein in your 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 active

# ITC Maintenance

Cell cleaning:

Water/buffer

20 % Contrad 70

Keep water in cell when not in use

Reference cell:

Replace water once a week

# Thank you!

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