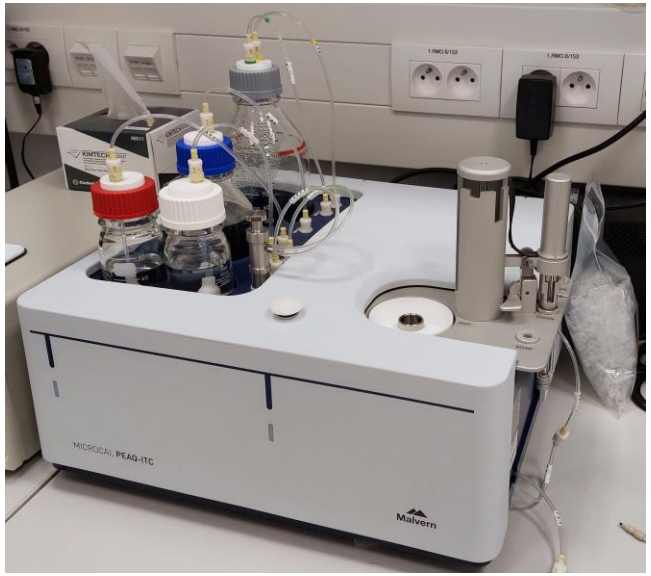

iTC experiment

Tatsiana Charnavets

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

Outline

- Experimental design
  - How much sample is required?
  - C value
  - Experiment optimization
- Sample preparation
- Data analysis
  - Evaluation of ITC raw data
  - Fitting models
- Troubleshooting
- iTC maintenance

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How much sample is required?

Do we have any information about the system? K_d value?

macromolecule [Cell] = 10 x K_d

ligand [Syringe] = 10 x [Cell]

Typically, macromolecular concentrations 10 – 100 µM (K_a ~ 10^2 - 10^9 M^{-1})

Min concentration macromolecular in the cell 10 µM
Min ligand concentration in the syringe 100 µM

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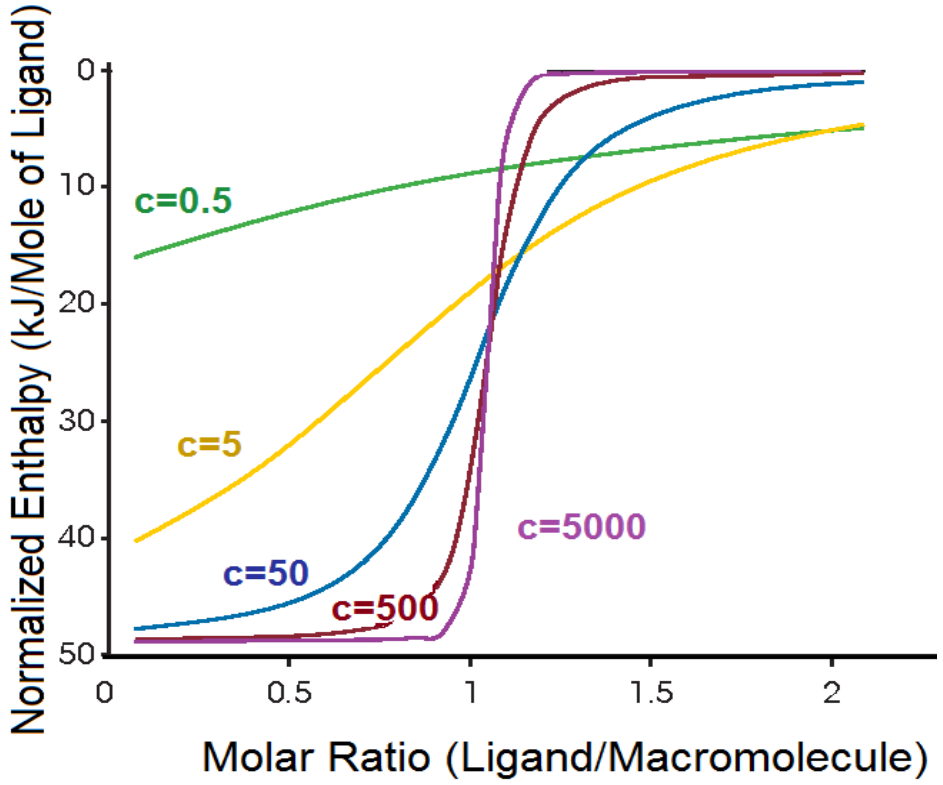
The shape of the isotherm varies according to the C value

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

for N=1; [M] is the concentration of macromolecule in the cell



If 1 < C < 1000, the three binding parameters can be precisely determined

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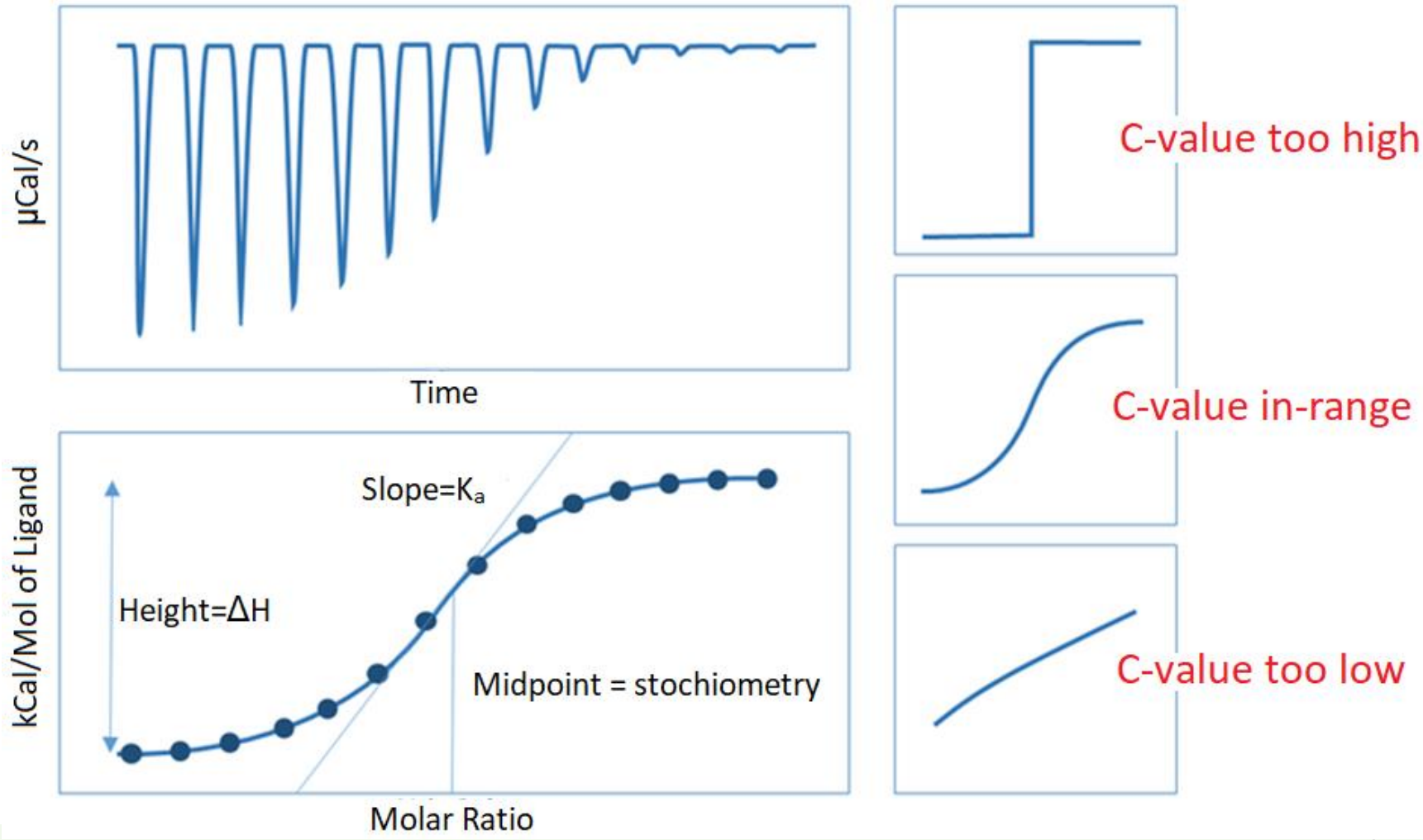
What happens if C-value outside of the optimal range?

$c > 10000$, only ΔH and N can be determined

$c < 1$, only K_d can be determined, as the ΔH and N will be correlated



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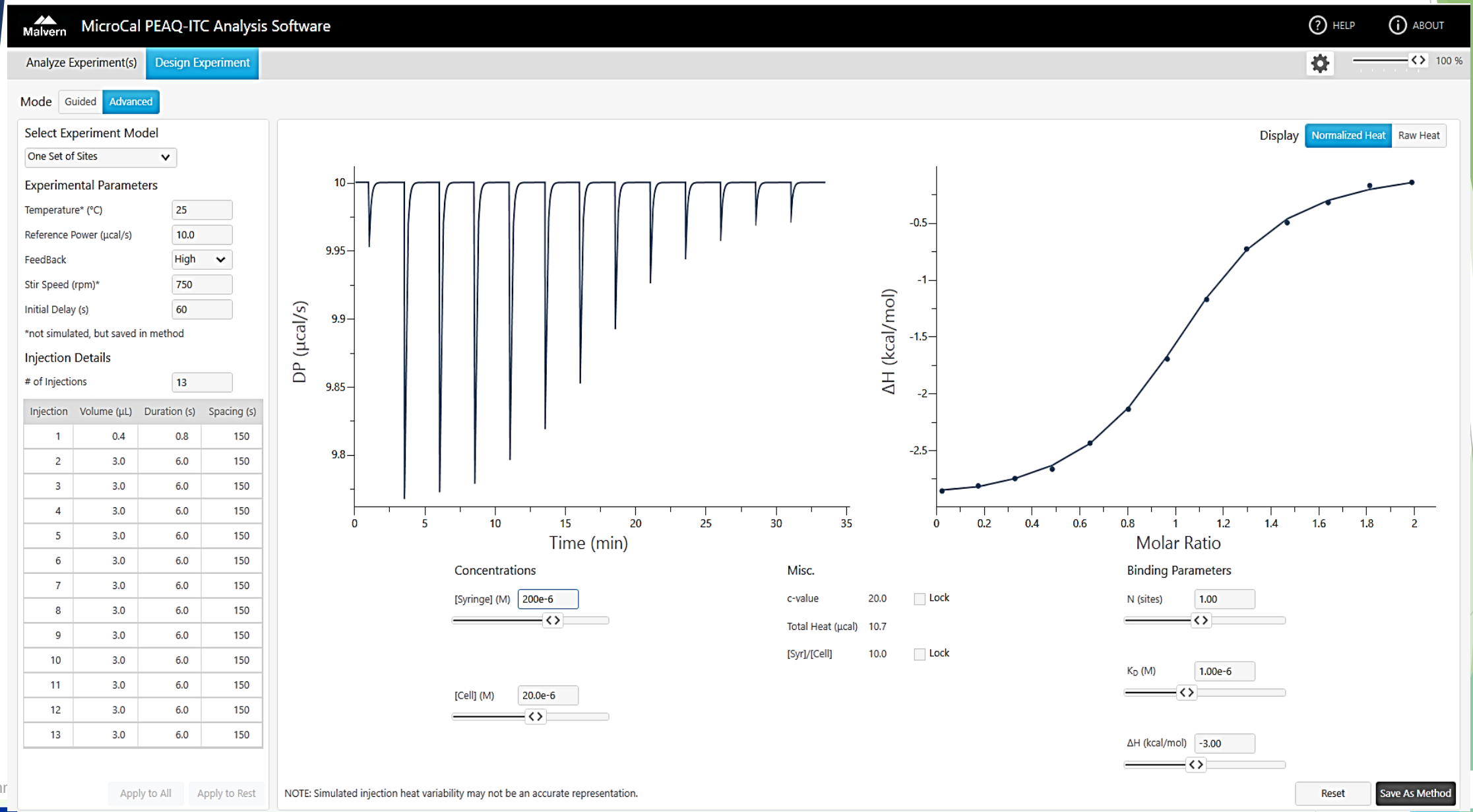
Aids experiment optimization saving time and sample



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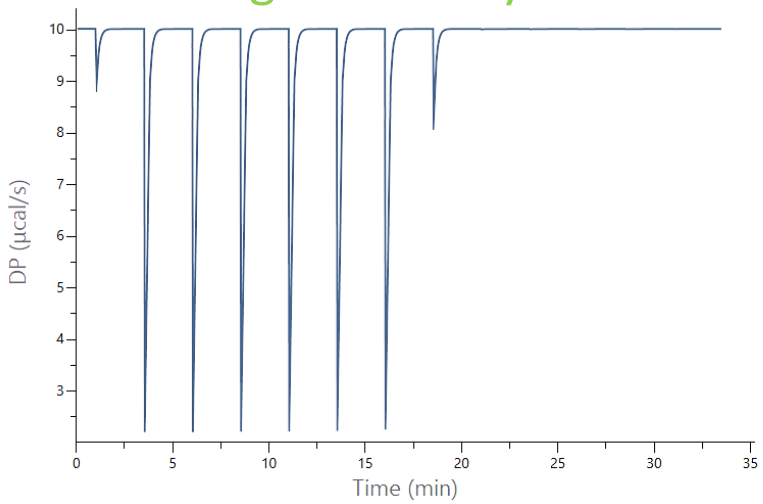
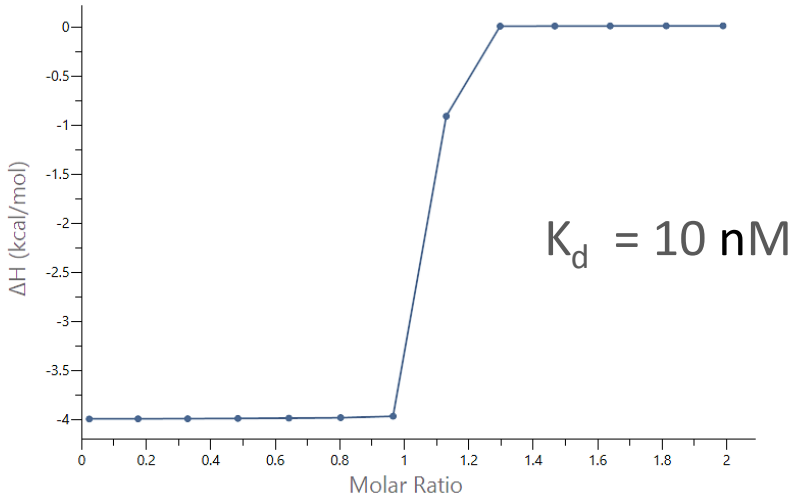
The effect of binding affinity on the shape of the titration curve

(1:1 stoichiometry)

Macromolecule and ligand concentrations were constant while K_d was varied

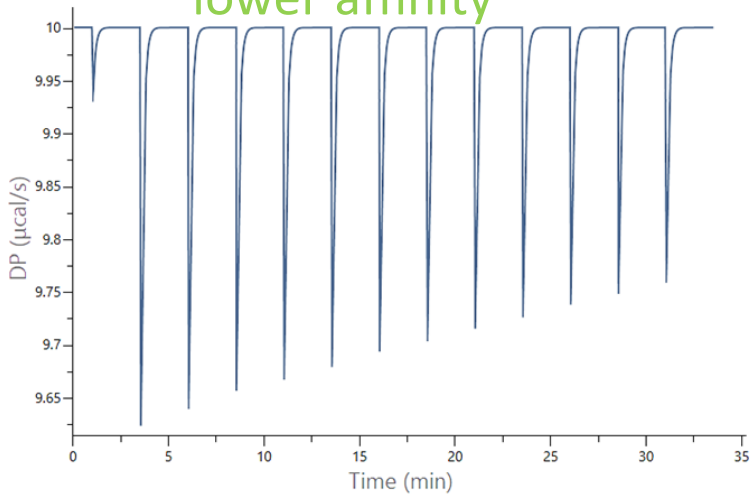
higher affinity

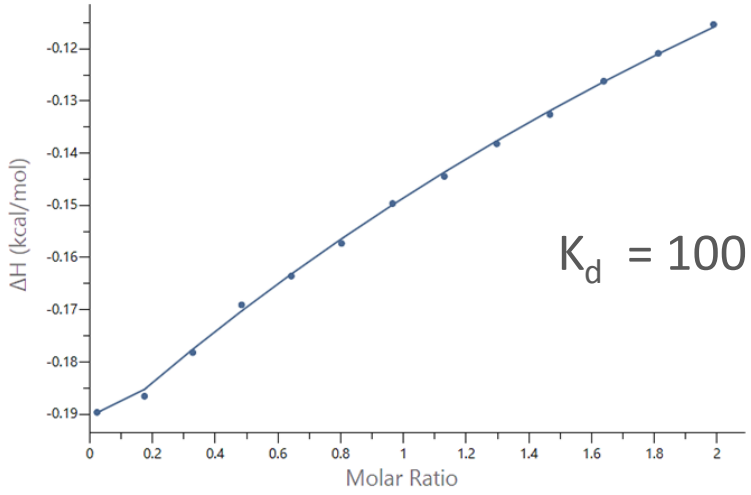




K_d = 10 nM

lower affinity





K_d = 100 µM

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Experimental design - creating the method

ITC200 - Taja

System ITC Options Help

Cells Heating To 25 °C | Time Left

Load Run File... | Save Run File... | Display Run Param. | Update Run Param. | Start | Stop

Experimental Design | Advanced Experimental Design | Instrument Controls | Real Time Plot | Setup

Experimental Parameters

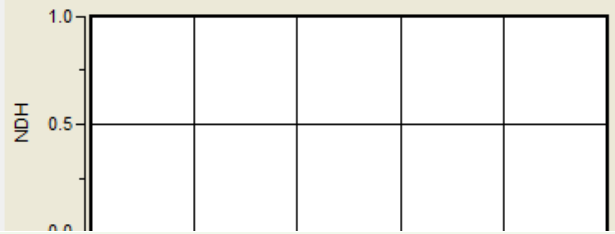
Total # Injections	20
Cell Temperature (°C.)	25
Reference Power (µCal/sec.) (0 - 12.25)	10
Initial Delay (sec.)	60
Syringe Concentration (mM)	5
Cell Concentration (mM)	0.4
Stirring Speed (0 - 2000 RPM)	750

Data File Name: default.itc

Feedback Mode/Gain: None | Low | High

Data File Comments...

Kd | dH | Update Experimental Curve

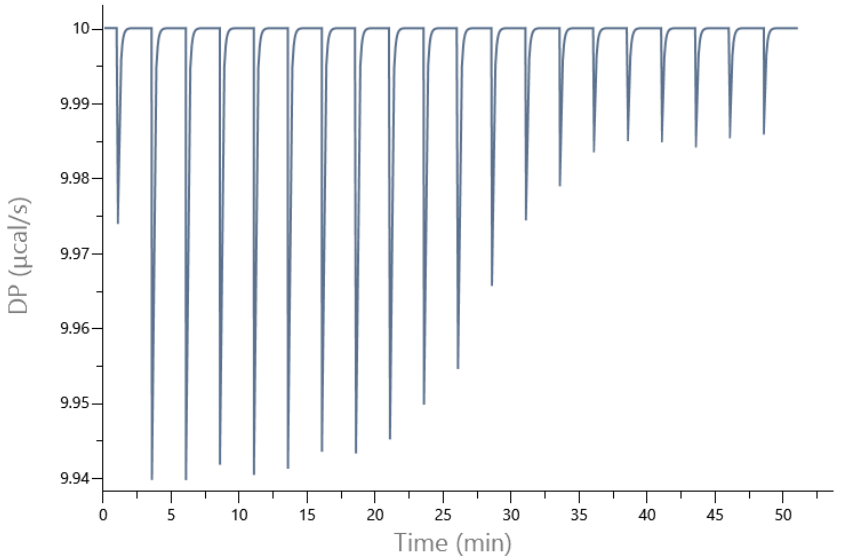


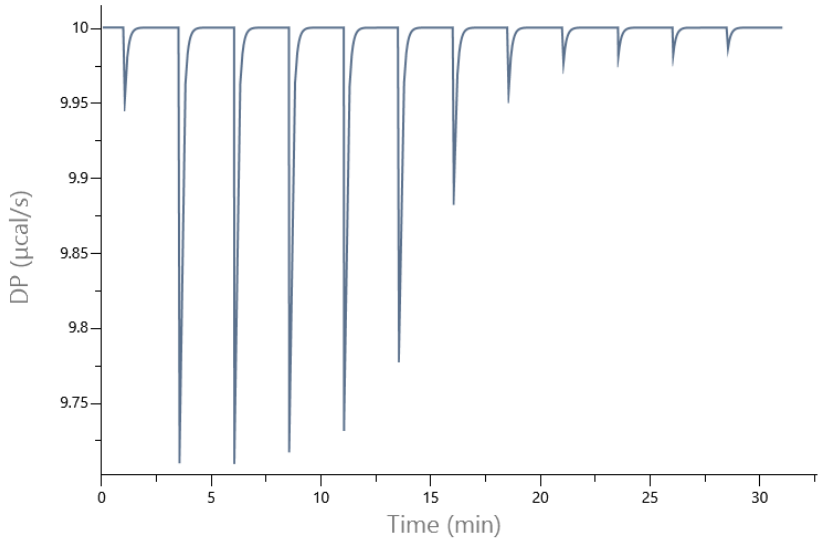
Injection Parameters

Volume (µl)	0.4
Duration (sec.)	0.8
Spacing (sec.)	150
Filter Period (sec.)	5

Edit Mode: All Same | Unique | Apply To Rest

	Volume	Duration	Spacing	Filter
1	0.4	0.8	150	5
2	2.0	4.0	150	5
3	2.0	4.0	150	5
4	2.0	4.0	150	5
5	2.0	4.0	150	5
6	2.0	4.0	150	5
7	2.0	4.0	150	5
8	2.0	4.0	150	5
9	2.0	4.0	150	5
10	2.0	4.0	150	5
11	2.0	4.0	150	5
12	2.0	4.0	150	5
13	2.0	4.0	150	5
14	2.0	4.0	150	5
15	2.0	4.0	150	5
16	2.0	4.0	150	5
17	2.0	4.0	150	5
18	2.0	4.0	150	5
19	2.0	4.0	150	5
20	2.0	4.0	150	5





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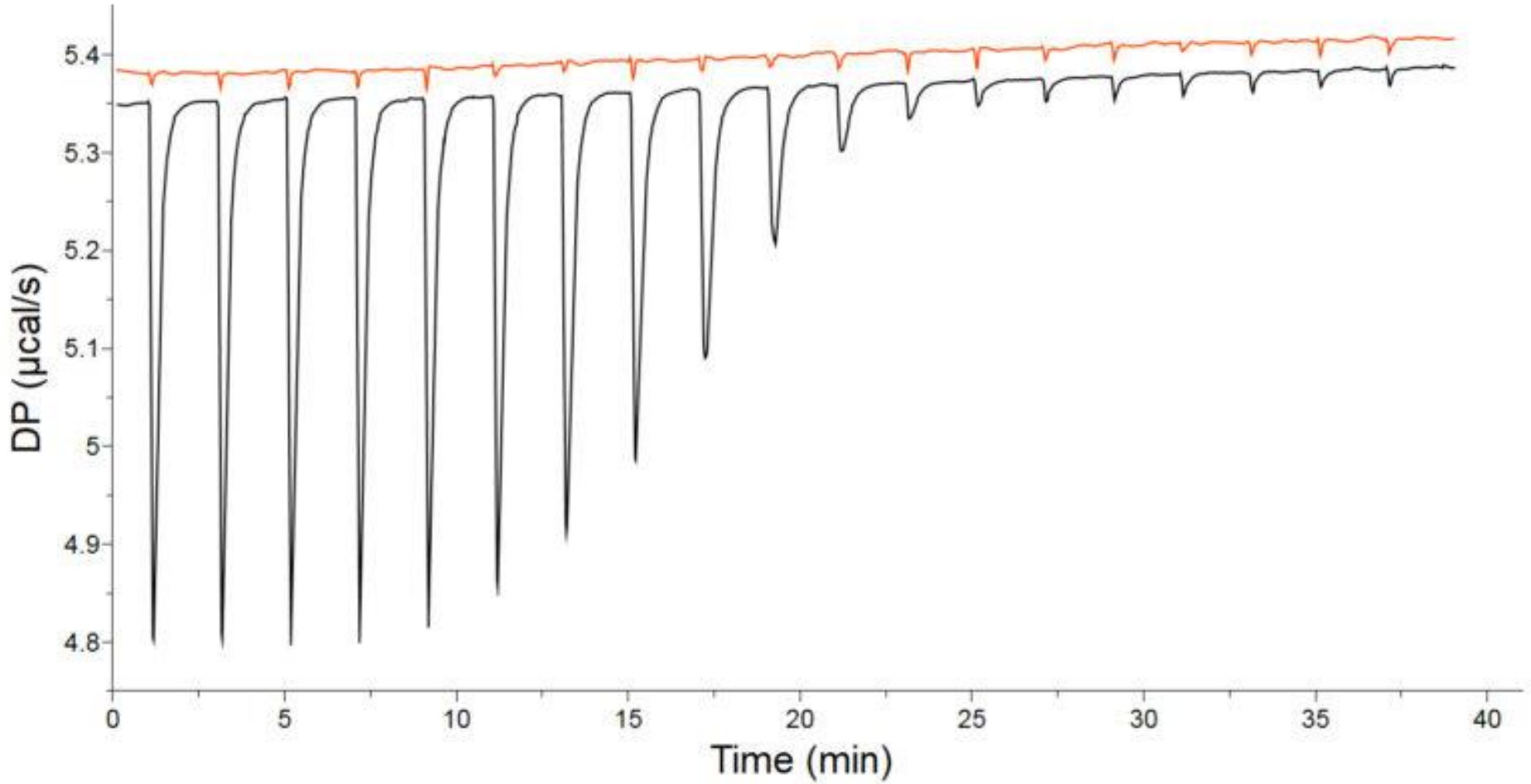
Reference Power and raw signal (DP) baseline



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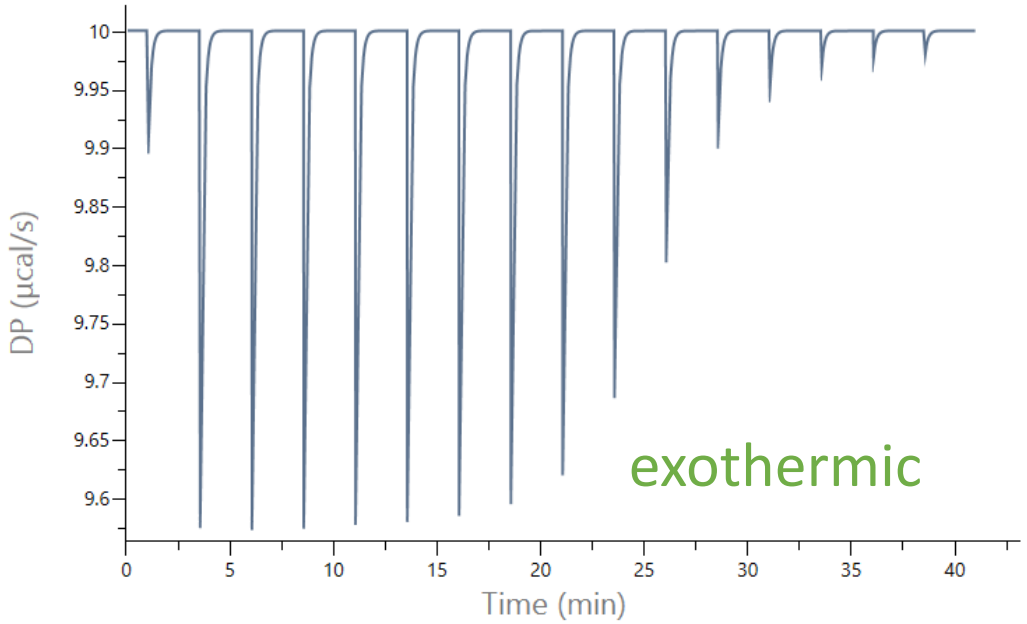
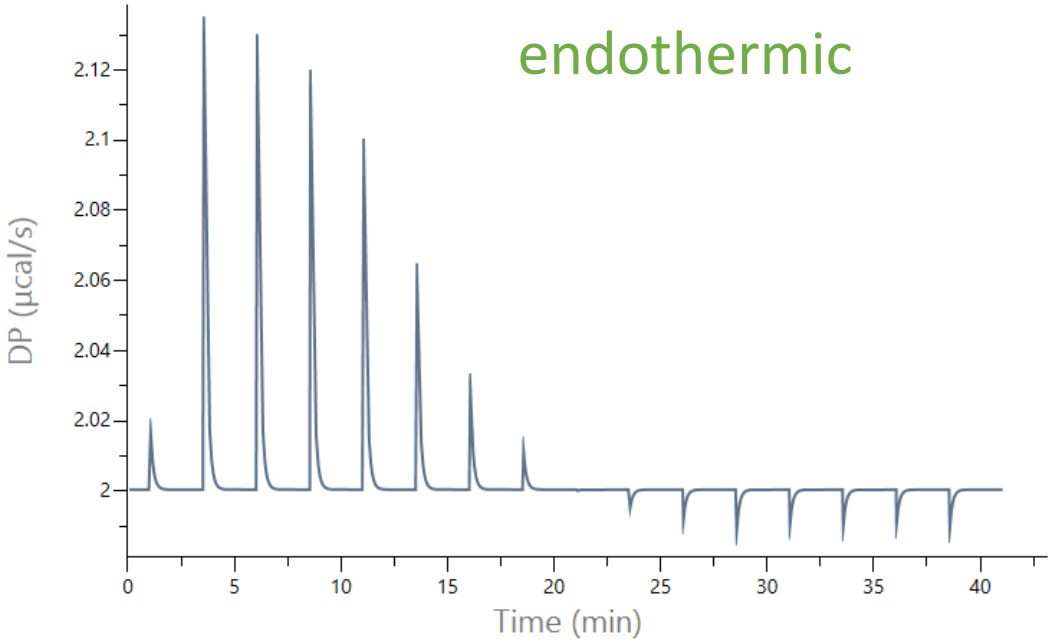
Choice for Reference Power setting





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Good sample preparation is key to ITC technique

Matched solvents

- Dialysis of the both macromolecules in the same buffer
- Using of dialysis buffer to make the ligand solution
- Ligand stored at high concentration (~100 mM) in DMSO
- Buffer exchange columns could be used

Centrifugation or filtration to remove any aggregated or insoluble material

Accurate molar concentration determination (errors in the cell concentration affect the stoichiometry; in the syringe concentrations affect n, K_d, ΔH)

Degassed macromolecular solution (5-10 min at 25°C with mixing)

Macromolecules and buffer are not directly from the freezer/fridge

Avoid air bubbles in the glass Hamilton syringe and in the sample cell

If you inject air bubbles into the sample cell, your experiment will not work!

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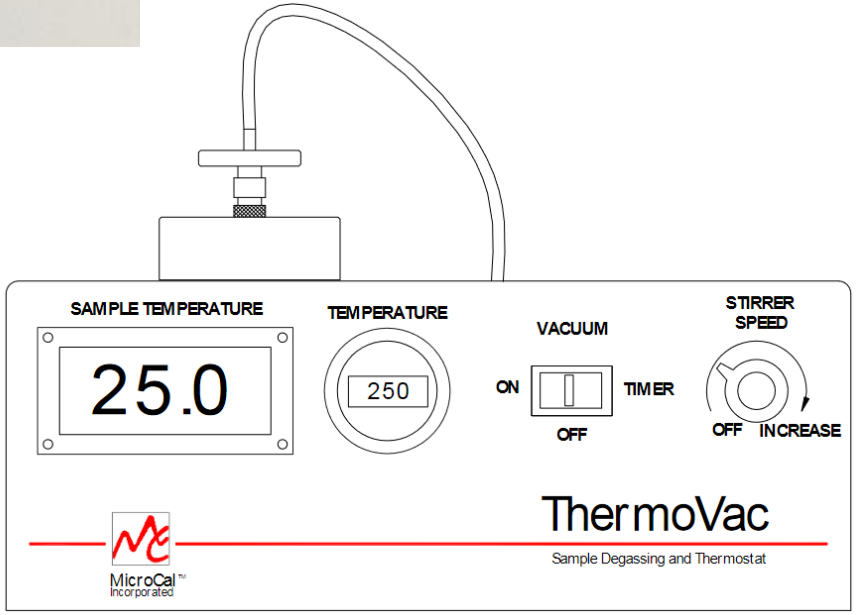
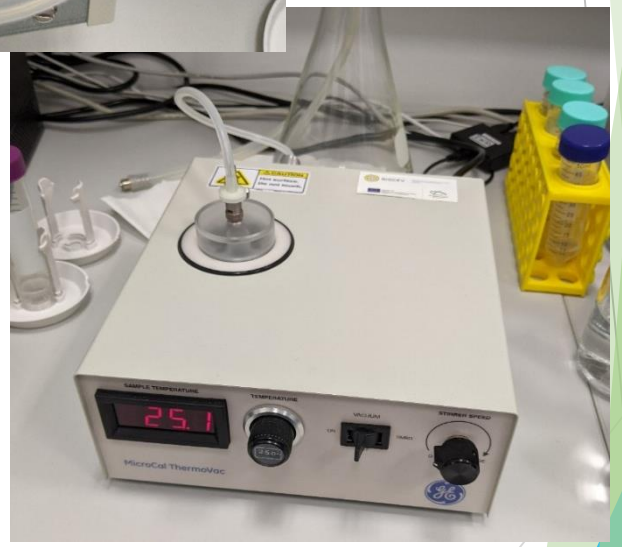
Sample degassing with ThermoVac

Thermostating
Stirring (magnetic stir bars)
Degassing





~ 5-10 min at 25°C and stirring for 300 µl of sample

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How to choose a good buffer

Identify the buffers in which the macromolecule and ligand are stable, taking into account pH and solubility

ITC cells are compatible with most buffers except strong acids

Sometimes additives are required for stability and solubility (e.g. salt, detergent or reducing agents)

Up to 10% DMSO in the buffer to improve the solubility of hydrophobic proteins

Glycerol displays large dilution heats – avoid, if possible

Sucrose, mercaptoethanol, TCEP, DTT, etc. - remember keeping at low concentration (<1 mM) and matched between all solutions, they cause baseline drift and high heats of dilution artefacts

Use buffers with a sufficient concentration to prevent pH effects

Use pH where they macromolecules and ligands are neutral

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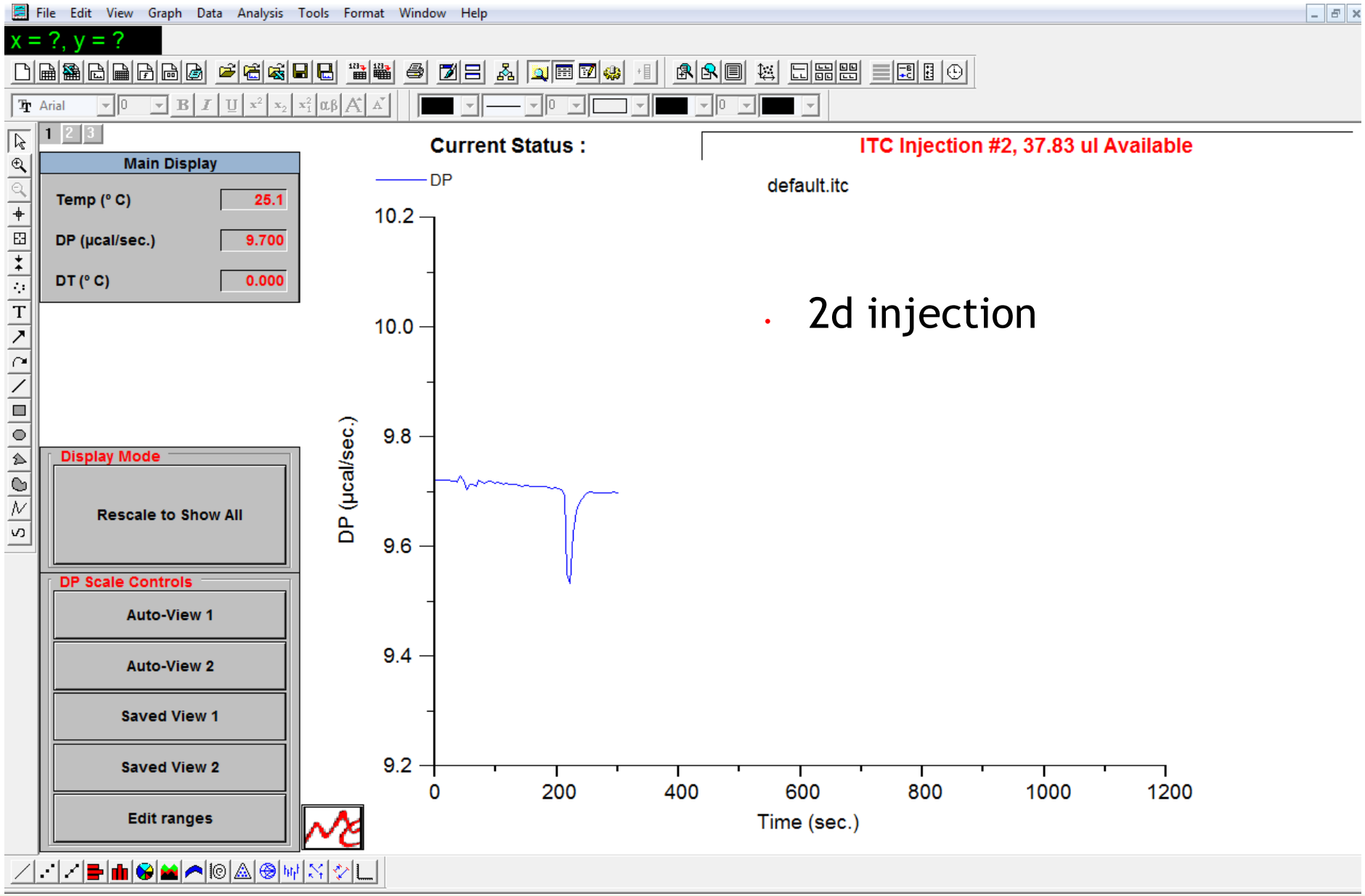
Titration run



- 2d injection

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Analysis of raw data to obtain N and ΔH

DH ΔH of each injection

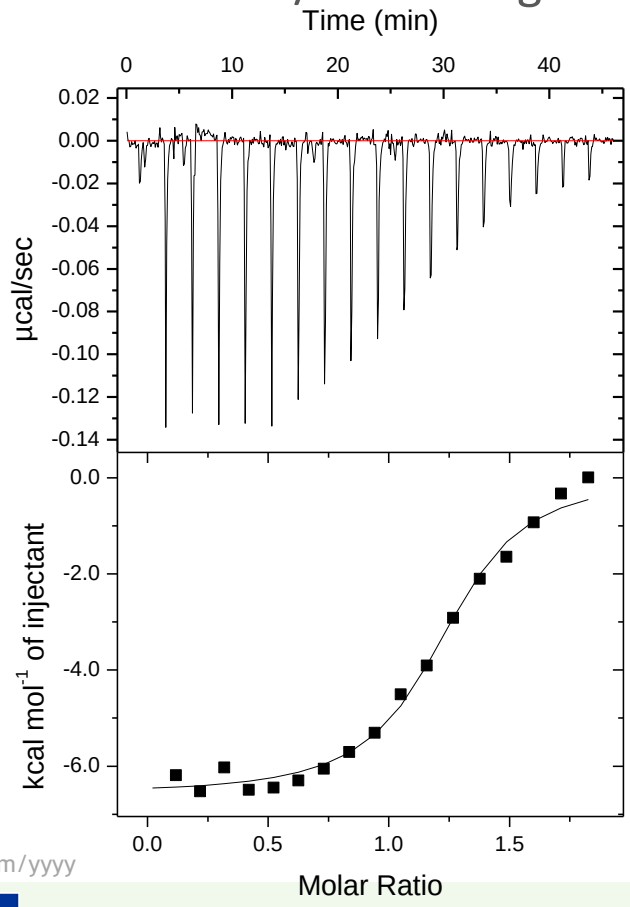
INJV Injection volume

X Concentration Ligand in the cell after each injection

Mt Concentration of the Macromolecule in the cell after each injection

XMt Molar Ratio (X/Mt)

NDH ΔH kcal/mole of ligand



default

	DH	INJV	Xt	Mt	XMt	NDH	DY	Fit
	[Y]	[Y]	[Y]	[Y]	[X]	[Y]	[Y]	[Y]
1	-0.44623	0.4	0	0.01	0.01962	–	–	-6451.7885
2	-1.25268	2	1.9579E-4	0.00998	0.11829	-6191.37011	245.47468	-6436.84479
3	-1.02053	2	0.00117	0.00988	0.21791	-6524.75962	-118.35796	-6406.40166
4	-1.24468	2	0.00213	0.00979	0.3185	-6029.93868	335.9723	-6365.91097
5	-1.34961	2	0.00309	0.00969	0.42005	-6492.96747	-181.88414	-6311.08333
6	-1.45786	2	0.00403	0.0096	0.52256	-6449.03846	-213.99369	-6235.04477
7	-1.33595	2	0.00497	0.0095	0.62603	-6299.76173	-173.33292	-6126.42881
8	-1.29894	2	0.00589	0.00941	0.73046	-6051.41933	-85.46695	-5965.95238
9	-1.24315	2	0.00681	0.00932	0.83585	-5708.5712	12.12837	-5720.69957
10	-1.17497	2	0.00771	0.00923	0.9422	-5303.19218	33.95239	-5337.14457
11	-1.02907	2	0.00861	0.00914	1.04951	-4508.65383	235.6235	-4744.27733
12	-0.92199	2	0.0095	0.00905	1.15779	-3907.61858	-4.70723	-3902.91134
13	-0.73653	2	0.01037	0.00896	1.26702	-2914.09521	-2.11932	-2911.97588
14	-0.58773	2	0.01124	0.00887	1.37721	-2103.33856	-100.0258	-2003.31276
15	-0.50884	2	0.0121	0.00879	1.48836	-1641.47979	-307.47216	-1334.00763
16	-0.38036	2	0.01295	0.0087	1.60047	-931.22704	-33.32845	-897.89858
17	-0.2734	2	0.01379	0.00861	1.71354	-327.87413	295.56349	-623.43762
18	-0.22037	2	0.01462	0.00853	1.82757	6.45317	454.81385	-448.36068
19		–	0.01543	0.00845	–			
20								
21								

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Fitting models

One-site

n ligands bind per macromolecule with identical thermodynamics

Two-site

model corresponds to two non-identical and independent (not cooperative) groups of ligand binding sites
In this case fit will generate n, K and ΔH for each site

Sequential

model corresponds to more than two ligand binding sites that might be identical or non-identical, independent or cooperative
1st ligand to bind to the macromolecule goes to the 1st site, and the nth ligand to bind goes to the nth site
It is a general model for any possible scenario with n ligand binding sites

Competition

two ligands with different affinities compete for the same binding site on a macromolecule
In a typical experiment to measure the Kd of a weakly binding ligand
Approximately 50% of the binding sites would initially be occupied by the weak binder
Than fill in the same binding sites by a ligand with a stronger affinity, displacing the weaker ligand

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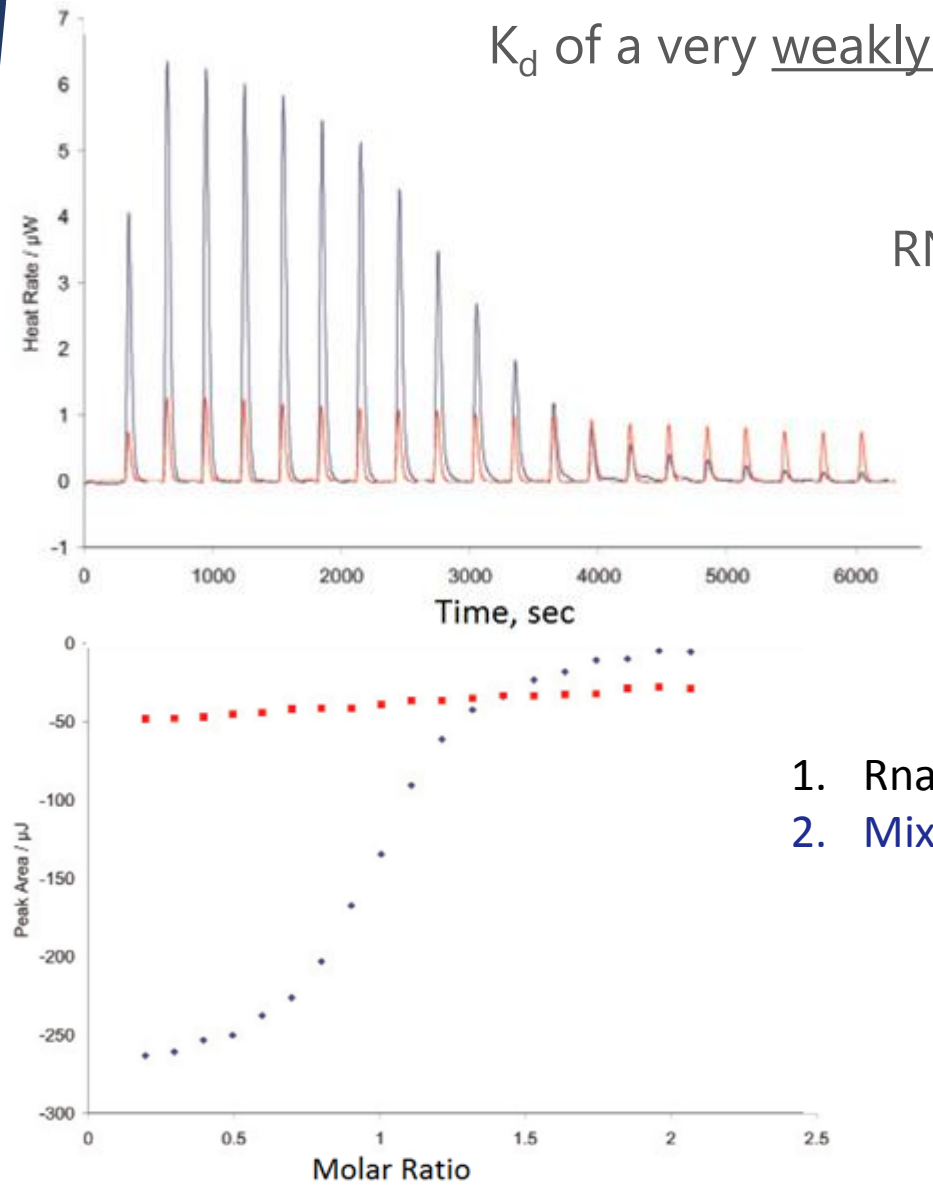
Competitive binding for the very weak or very tight association constants

K_d of a very <u>weakly binding ligand 5′-CMP to RNase?</u>



RNase binds both 2′-CMP and 5′-CMP in the same binding place, but with different binding affinity

1. Rnase [70 µM] was prebound with weaker binder 5′-CMP [0.32 µM]
2. Mixture was titrated by stronger binder 1.3 mM 2′-CMP

⇒ Kd for 5′-CMP binding can be accurately calculated

https://www.tainstruments.com/pdf/literature/M123.pdf

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N-value is a measure of the binding ratio as much as the activity of the samples

✓ Stoichiometry

concentrations of molecules we use for the fitting are correct

molecules 100% active)

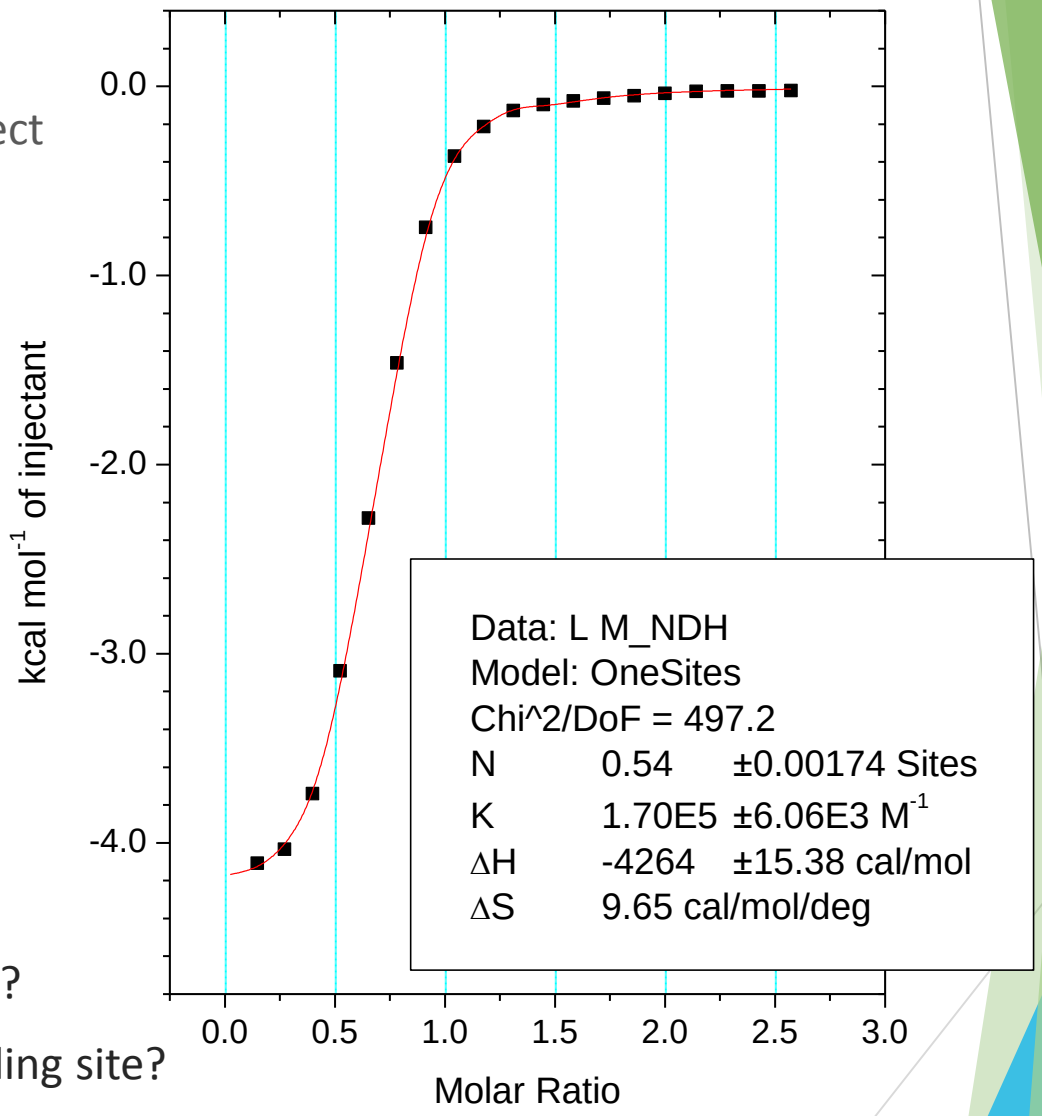
✓ Binding activity of samples

$$N = \frac{[\text{Active fraction in the cell}]}{[\text{Active fraction in the syringe}]}$$

N = 0.5

macromolecule is a dimer and binds one ligand per dimer?

ligand has 2 binding sites, and the sample in the cell has one binding site?



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Optimization of ITC experiments

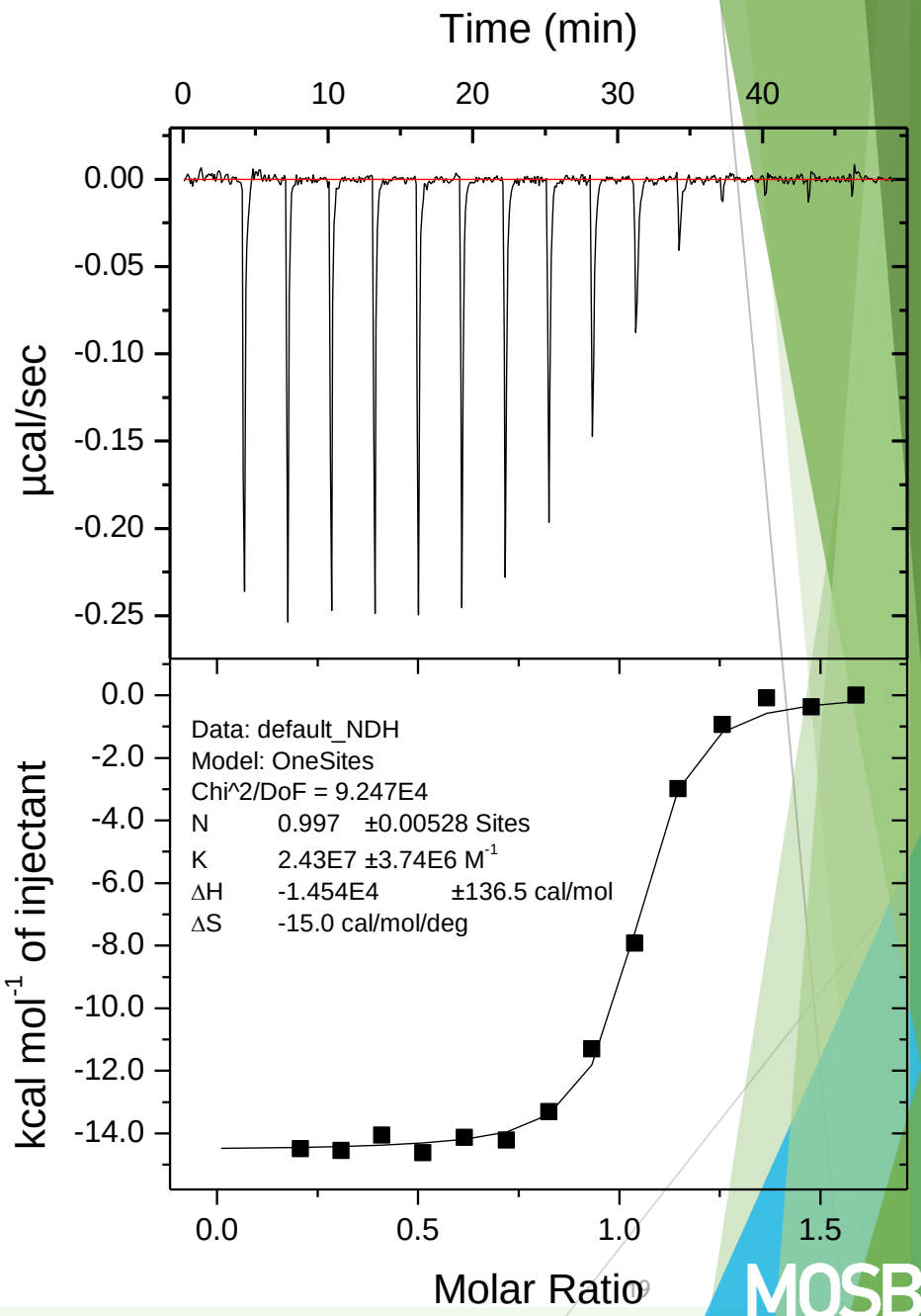
syringe ligand concentration → N, Ka and ΔH

cell macromolecule concentration → N

If the ligand concentration there is not high enough to achieve binding saturation:

keep the macromolecule-ligand complex in the ITC cell and perform a second titration experiment

This can be repeated until binding saturation is reached



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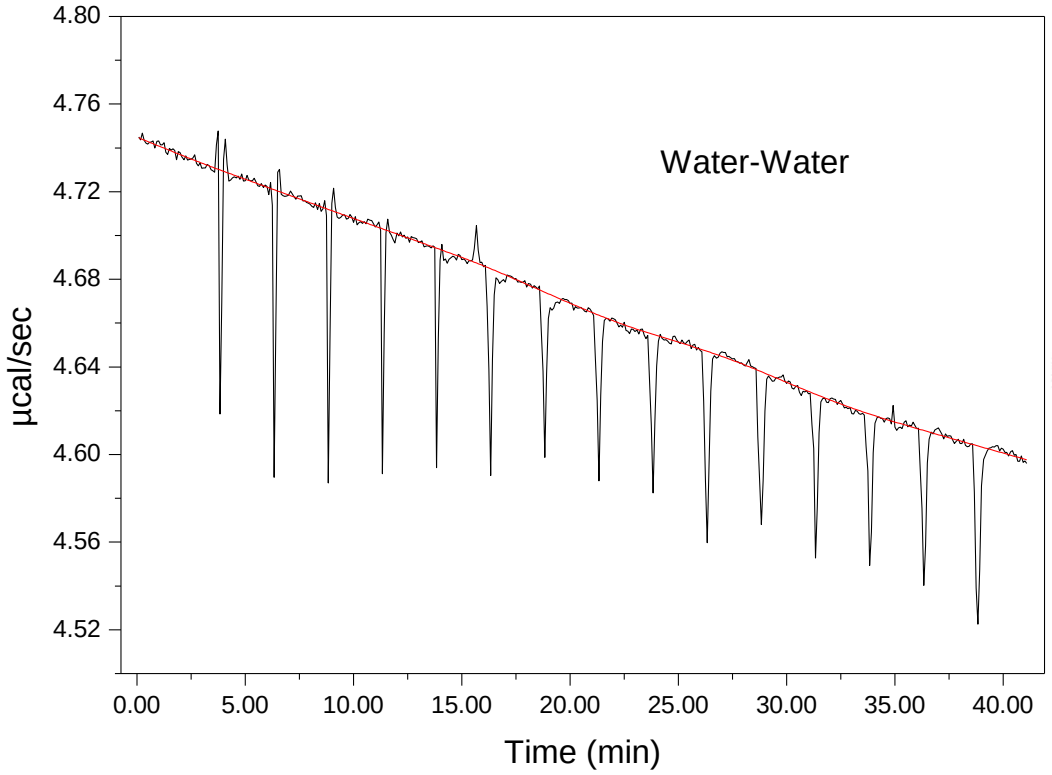
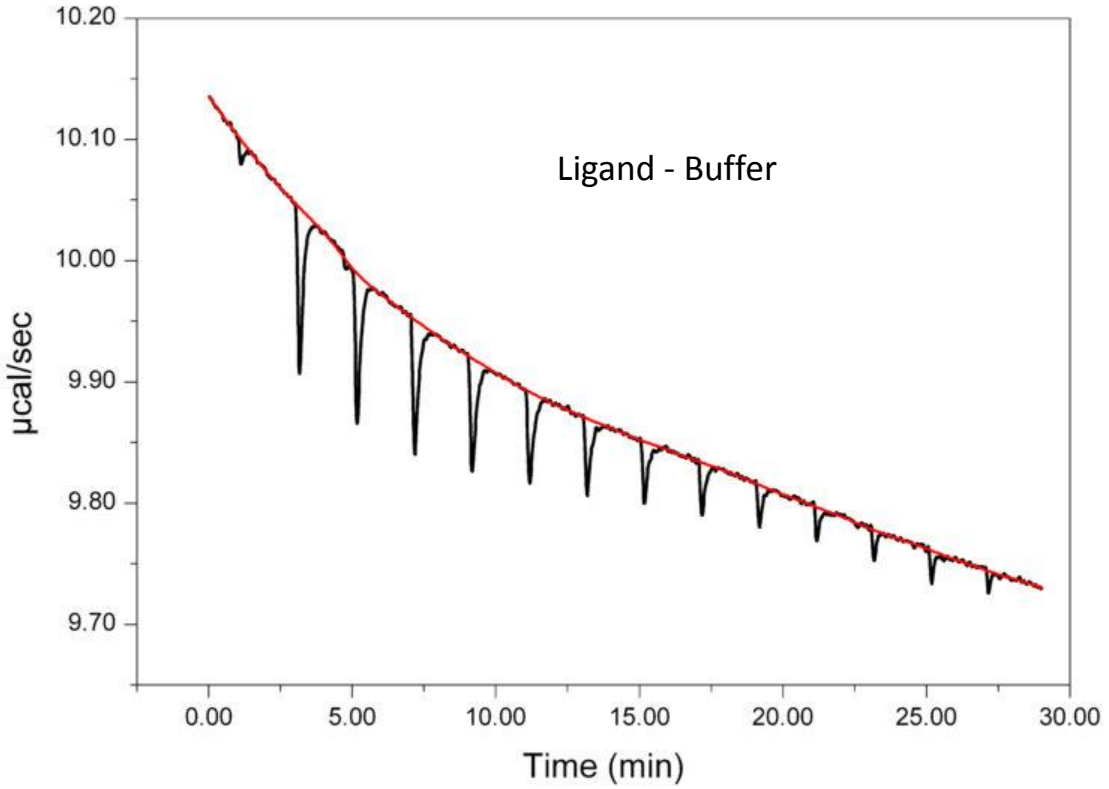
Control titrations (water/water, buffer /buffer, ligand/buffer)

reproducible heats of injection throughout the titration

plot with no or a shallow slope





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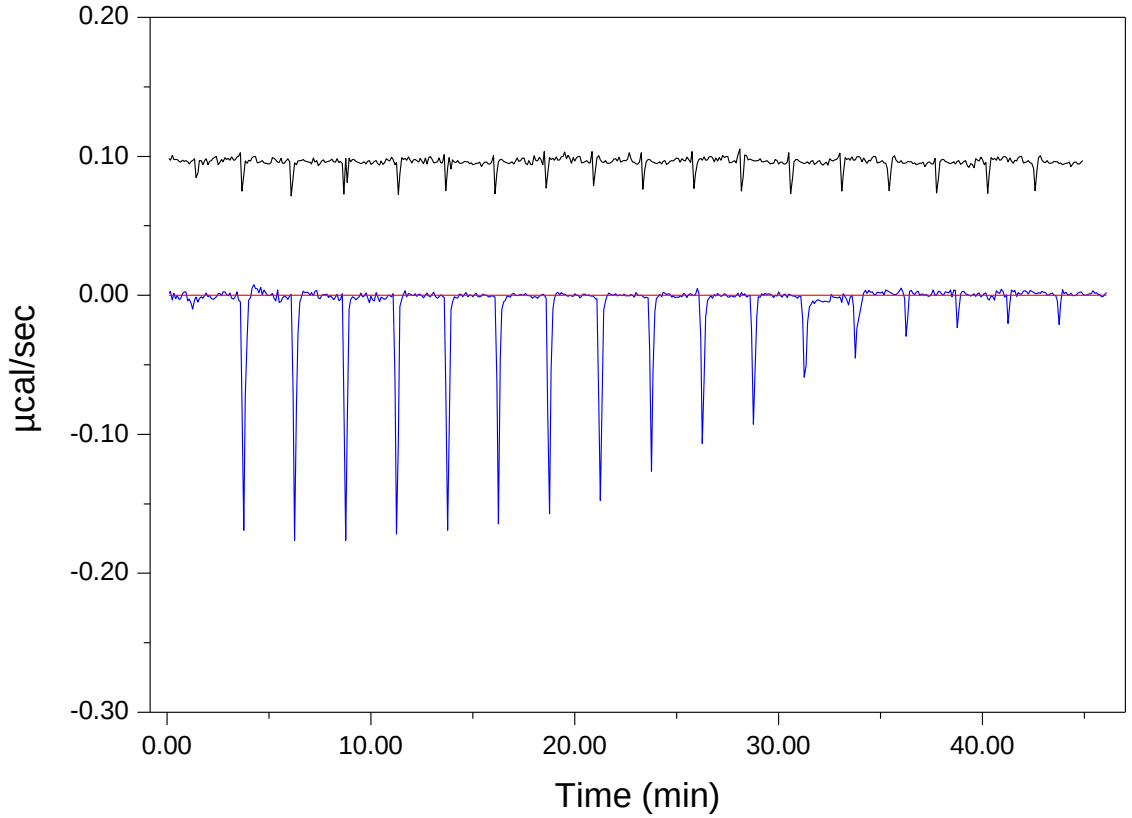
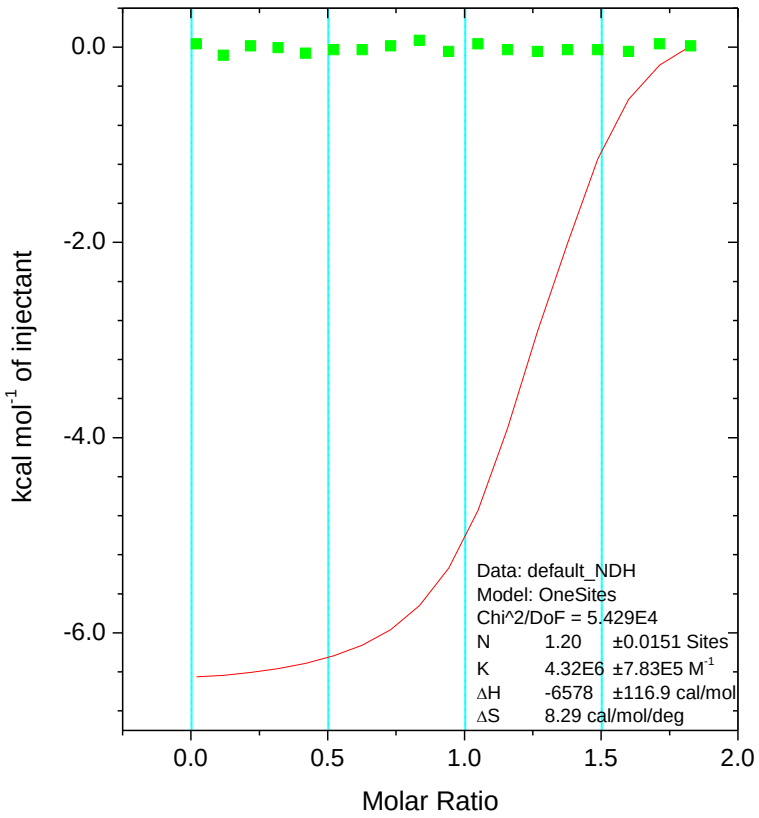
Correcting for the heat of dilution, prior to data analysis





Control peaks should be small and equal in magnitude to one another

Peaks at the end of the titration experiment should be similar in magnitude to those in control

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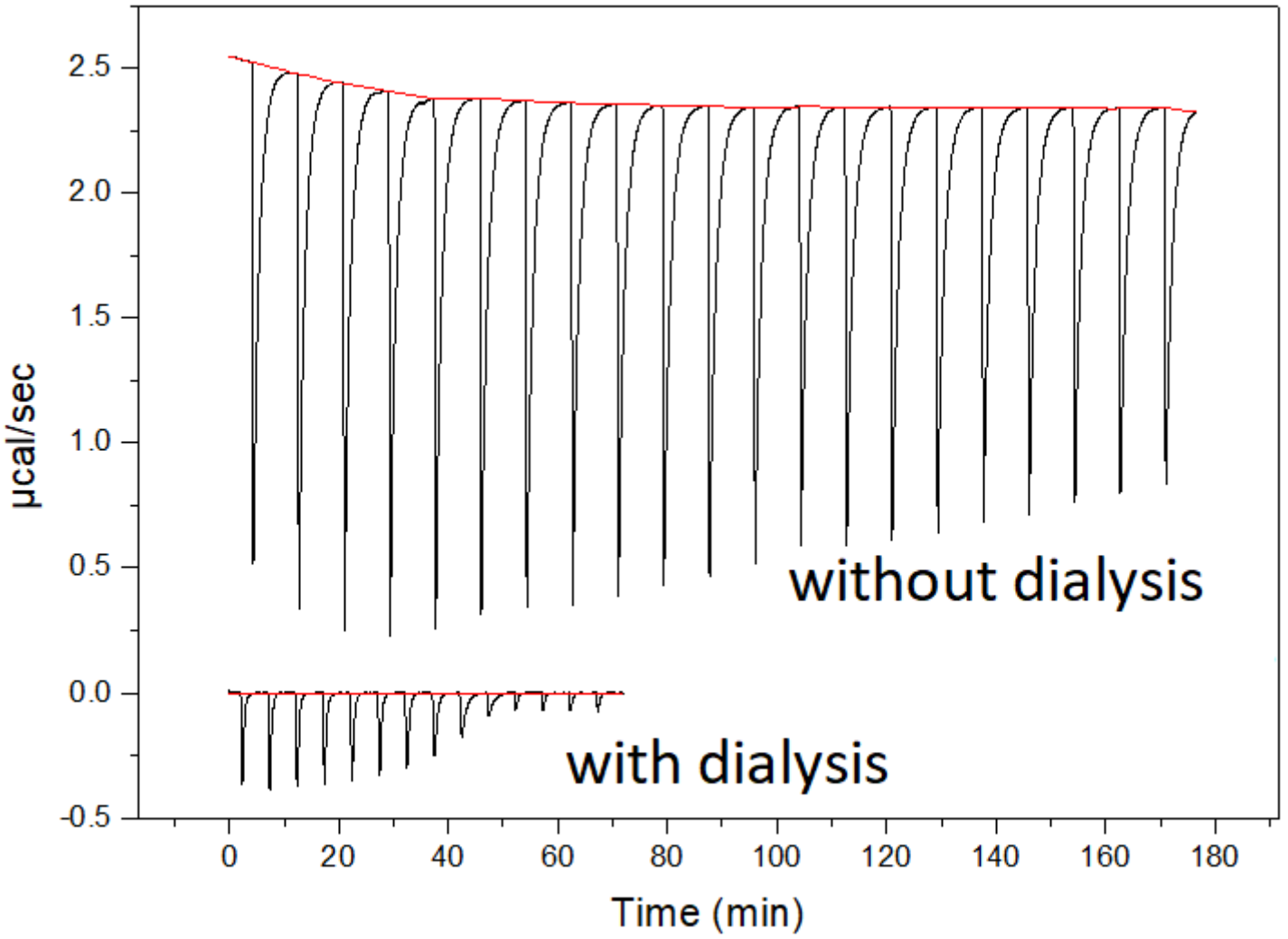
Sample preparation – dialysis for the solvent matching

- ✓ Final dialysis buffer can be used to make up the ligand solution
- ✓ Both the macromolecule and the ligand might be dialyzed in the same buffer



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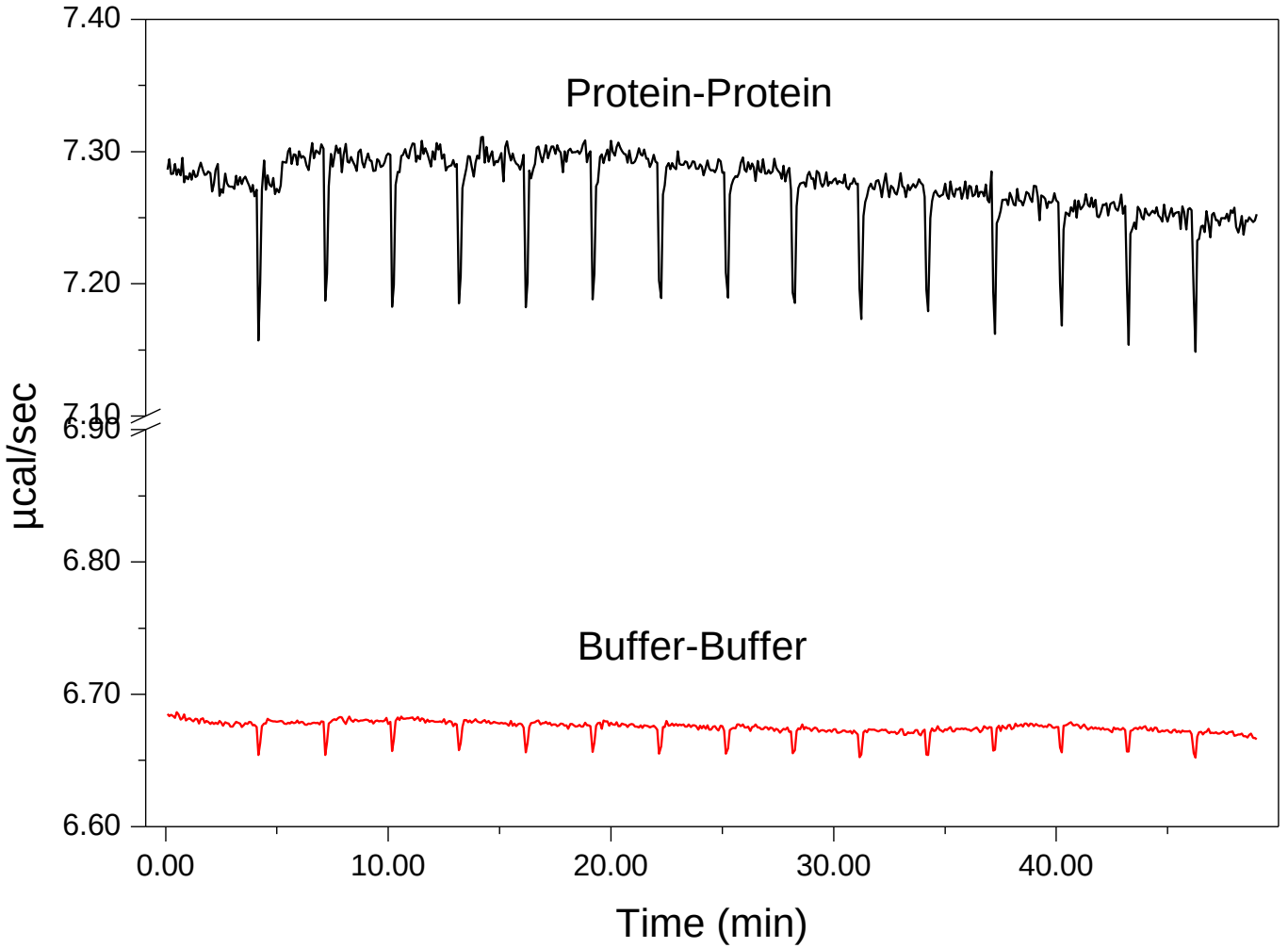
Noisy baseline and/or spikes between injections



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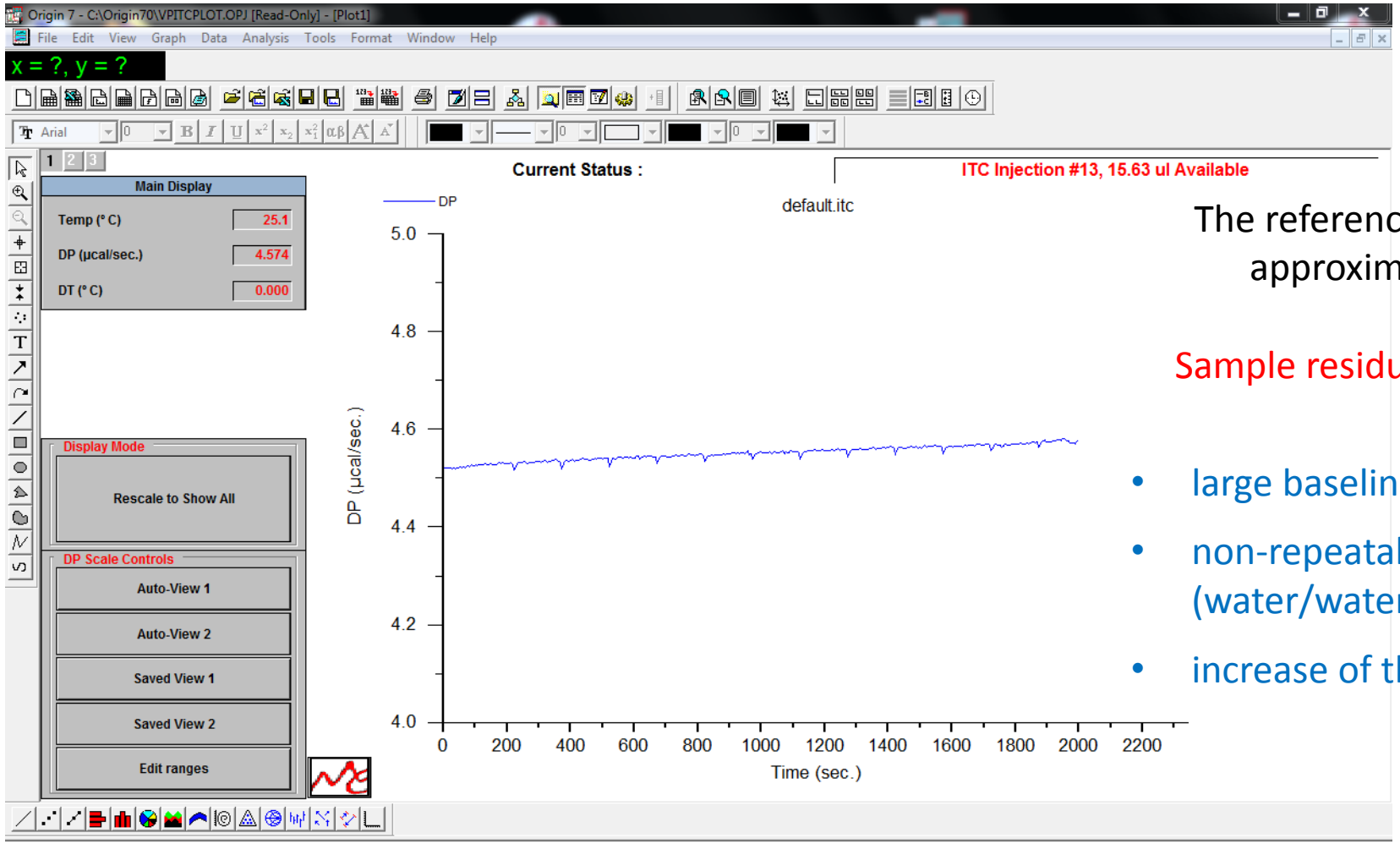
iTC maintenance



The reference cell must be refilled approximately once a week

Sample residue will cause problems!

- large baseline drifting
- non-repeatable control peaks (water/water)
- increase of the noise level

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Common issues with ITC raw data and possible causes

- Large injection heats due to the large heats of dilution ! buffers mismatch !
- DP does not return to pre-injection baseline before the next injection ! insufficient time between injections !
- Spikes in injections, some peaks are much larger or much smaller than others ! air bubbles !
- Noisy baseline and/or spikes between injections
- DP value significantly different than RP ! bent injection needle !
- Stepping baseline: there is a change in the heat capacity of the system
- No detectable binding heat ! no binding or binding enthalpy is lower than expected !
- The binding isotherm looks like a straight line, with no evidence of binding saturation ! concentrations of the cell and the syringe sample!

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Syringe and cell wash - with washing module

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Recommended cleaning reagents

Rinsing the ITC cell after each ITC experiment with

20% Contrad 70

14% Decon 90,

followed by water

Perform regular “soaks” with 20% Contrad 70 in the ITC cell, heated to 60 °C, for 30 minutes

After the cooling the cell, rinse it with water

For the sticky proteins you may need to do more frequent detergent soaks

Rinse the ITC cell with sample buffer, before filling the iTC cell with sample

Make sure the ITC syringe is rinsed with methanol and dried before adding the ligand

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Isothermal titration calorimetry for the biomolecular interaction analysis

tatsiana.charnavets@ibt.cas.cz

http://www.ibt.cas.cz IBT web page

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