

## ITC Sample Preparation Guidelines

*For VP-ITC,*

≥ 1.8 ml of protein for sample cell (1.4 mL cell + 0.4 mL dead volume)

≥ 500 μL of ligand for syringe (300 μL syringe + 200 μL for filling);

*For iTC200 (7 × less volume; 2.5 × more sensitive),*

≥ 300 μL of protein for sample cell (202 μL cell + ~ 80 μL dead volume)

≥ 60 μL of ligand for syringe (40 μL syringe + 20 μL for filling)

Typical starting concentrations are 10-50 μM for the cell and 50-500 μM for the syringe.

See “*Experimental Design*” below to estimate suitable concentrations for your particular experiment.

*General points,*

- syringe concentration ideally 10 x higher than molar concentration of protein in cell, 7 x higher for very tight binding, 15 - 20 x higher (or more) for weak binding (Since the syringe volume is ~ 5 fold the cell volume, a 10 fold excess in the syringe will give a final ~ 2 fold excess in the cell)
- molar concentrations *must* be known accurately. If using extinction coefficients, consider correcting for scattering ( $A_{280}(\text{true}) = A_{280} - (1.96 \times A_{330})$ ; Pace *et al.*, (1995) *Protein Science* 4:2411-2423), and calibrating them against e.g. amino acid analysis assay. Errors in the cell concentration affect the stoichiometry ( $n$ ); errors in the syringe concentration are more problematic as they affect  $n$ ,  $K_a$  and  $\Delta H$ . Remember that unfolded/inactive components will reduce your effective vs measured concentration
- for previously untried systems prepare larger volumes than the above for additional runs at different concentrations and control experiments
- absolutely no bubbles, floating fibres or precipitate (i.e. spin down hard or filter)

*Buffers,*

- both protein and ligand must be in identical buffers or large heats of dilution (background heats) may mask the desired observation. Exhaustive dialysis of both components in the same container is ideal, or for small-molecule ligands use the final dialysis/flow-through buffer to make up the ligand solution after thorough desalting
- keep ~50 mL of dialysis/flow-through buffer for rinsing, baselines, dilutions etc
- the Hastelloy alloy that ITC cells are manufactured from is compatible with most buffers except strong acids; detergents and/or organic solvents may be incorporated as well provided appropriate precautions are taken to minimize heat of dilution artefacts, etc.
- if you have very high ligand concentrations (mM), make sure your buffer concentration is high enough to compensate for any pH swing and check the pH beforehand
- reducing agents such as DTT cause erratic baseline drift and other artefacts. TCEP is recommended over β-mercaptoethanol. Avoid if possible or keep ≤ 1 mM if absolutely required, especially if  $\Delta H$  is small

- bear in mind that different buffers have different ionisation heats so if your reaction involves e.g. protonation  $\Delta H$  can vary widely between different buffers (see tables in "Resources" section of Biophysics webpages; phosphate, citrate, acetate have low  $\Delta H_{ion}$  and thus give minimal additional heat contributions)
- if DMSO is required to solubilise the ligand, add DMSO to the macromolecule solution to match immediately before running the experiment. The recommended procedure is to make up the same volume of syringe and cell solution, then add the same volume of DMSO to each using the same pipette. Any small mismatch will give very large heats of dilution. Many proteins are stable in the short term in up to 2-5% DMSO (DMSO may reduce the measured  $K_d$  as it competes for binding, to correct  $K_d$ , measure  $K_d$  of protein + DMSO in a separate experiment)

## Experimental design

*Concentrations to aim for if  $K_a$  (or  $K_d$ ) is known,*

- the critical parameter is the dimensionless sigmoidicity constant,  $c$ , calculated as follows:

$$c = n \times K_a \times M_{cell} = n \times M_{cell} / K_d$$

where  $n$  is the stoichiometry and  $M$  represents concentration, expressed in the same units as  $K_d$

- aim for a  $c$  value of 10-50 (10-20 for iTC200) to ensure an S-shaped titration curve with a plateau. Higher  $c$ -values result in titration curves that are too steep to resolve  $K_a$  accurately (although  $n$  and  $\Delta H$  are well determined) because the cell concentration is too high relative to  $K$ , whereas lower  $c$ -values result in shallow titration curves from which all three parameters ( $n$ ,  $K$ , and  $\Delta H$ ) are poorly determined.

- another key factor is  $\Delta H$  which should be at least 10  $\mu\text{cal}/\text{injection}$  at the start of the experiment; the injected volume can be adjusted to attain this level if necessary; consider also optimizing the magnitude of  $\Delta H$  by adjusting the temperature (typically  $\Delta C_p (= \Delta H/T)$  for biological reactions is 0.3 to 2  $\text{kcal}/\text{mol.K}$  and is almost always negative; many systems have a  $\Delta H/T$  that crosses zero between 20-30  $^{\circ}\text{C}$ ) or the buffer (see effects of  $\Delta H_{ion}$  above)

*If  $K_a$  (or  $K_d$ ) is NOT known,*

- start with e.g. 50  $\mu\text{M}$  ligand in syringe, 5  $\mu\text{M}$  protein in cell, if peaks are too small, increase concentrations

*If  $K_a$  (or  $K_d$ ) is approximately known and you know  $n$ ,*

- perform a range-finding experiment starting with e.g.  $M_{cell} = 10$  to  $50 \times K_d$  and  $M_{syringe} = 10 \times n \times M_{cell}$

*If the binding is very tight,*

- using an optimal  $c$  value of 10-50 will result in low values for  $M_{cell}$  and  $M_{syringe}$  that will produce a very small  $\Delta H$
- the VP-ITC with its higher volume capacity is more sensitive for low  $K_d$ s

- consider optimising  $\Delta H$  by changing temperature or buffer
- where  $K_d$  is too small to measure accurately, consider doing competition experiments using an excess of a weaker-binding ligand (see example in “Resources” section of Biophysics webpages)

*If the binding is very weak,*

- using a  $c$  value of 10-50 will result in very high values for  $M_{\text{cell}}$  and  $M_{\text{syringe}}$  that may not be attainable with the amount of available material or may produce highly non-ideal solutions
- having  $M_{\text{syringe}} \gg M_{\text{cell}}$  is important to drive the equilibrium towards binding
- do not stop the titration early, try to achieve a plateau in the isotherm (70-90% saturation can be fitted if  $n$  is known and fixed)
- an experiment that does not plateau may be continued by refilling the syringe, leaving the cell intact, and starting a second run; use “concat.exe” on the Desktop to join the two .itc files together to produce a single data file
- bear in mind that  $\Delta H$  will not necessarily be well determined
- aim for syringe:cell molar ratio of 1:1 after 10 injections, 4:1 or higher by the final injection
- consider doing competition experiments using a stronger-binding ligand

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