OPPF

Oxford Protein Production Facility

The Thermofluor Method

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PEPC5

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MRC Medical Research Council

STRUCTURAL PROTEOMICS SPINE IN EUROPE

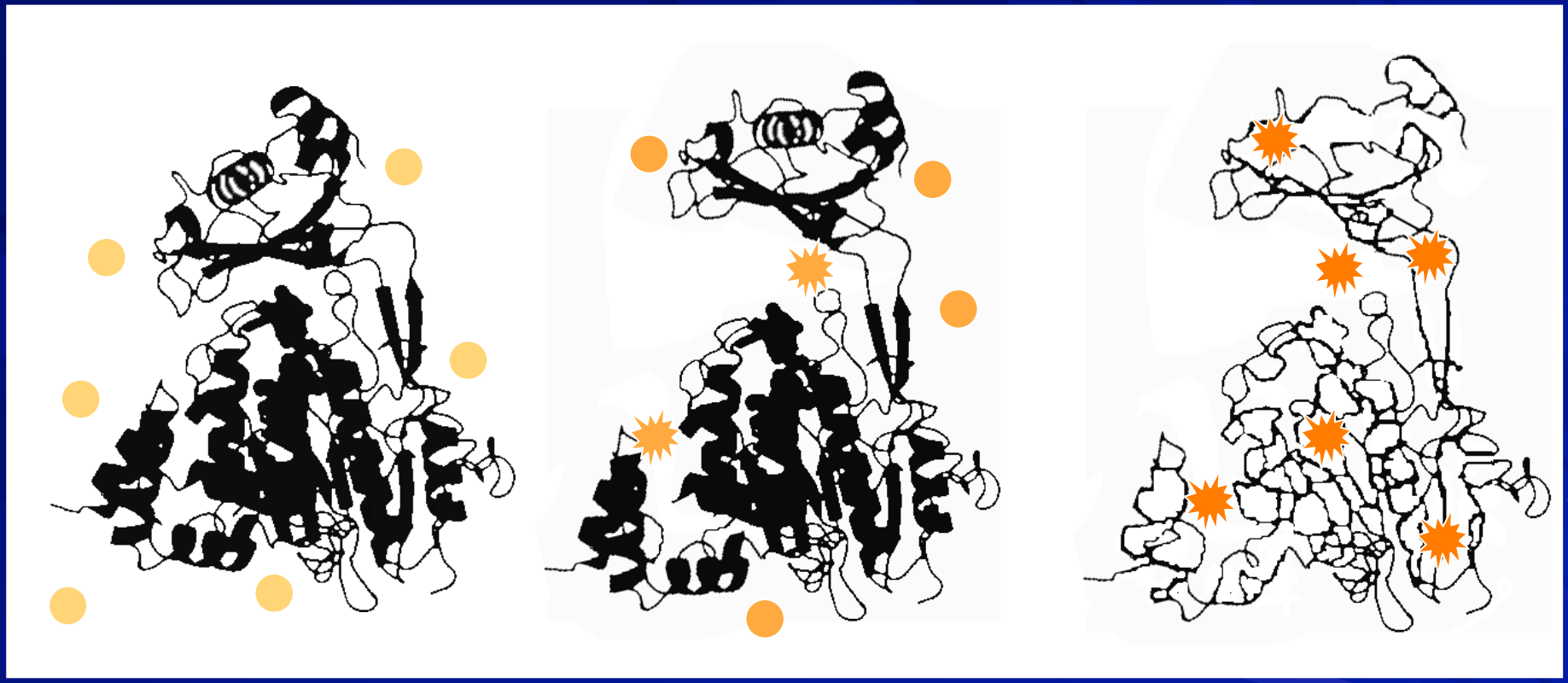
Vizier

- Background and Methodology
- Design and use of a general screen
- Focussed ligand-binding assays
- Limitations

Background

- A biophysical technique used to study (relative) protein stabilities
- The solution is heated stepwise from room temperature to ~95°C and fluorescence is monitored at each step
- Rising temperature causes protein unfolding and the fluorophore (SYPRO Orange) partitions itself into the melted protein and hence the overall effect is *an increase in fluorescence with increasing temperature*
- If a drug/ligand is included which binds to the protein, the mid-point of the melt curve can shift, indicating stabilising or destabilising effects (e.g. Ligand binding)

Increasing temperature



Folded Protein

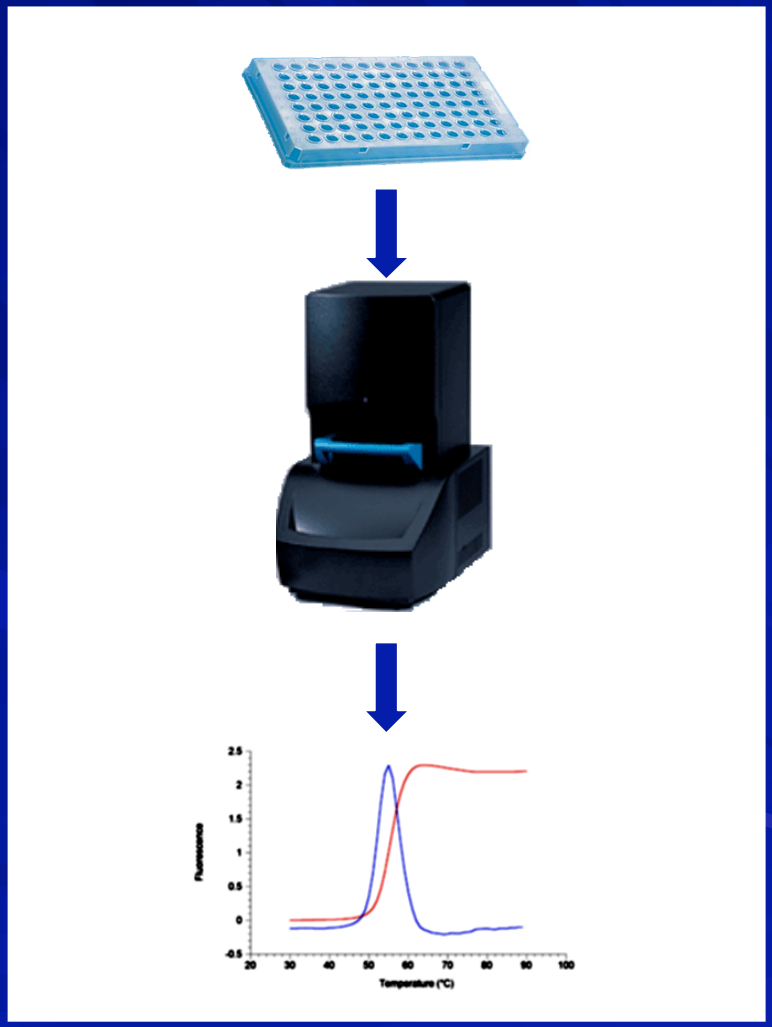
Partially Unfolded Protein

Unfolded Protein

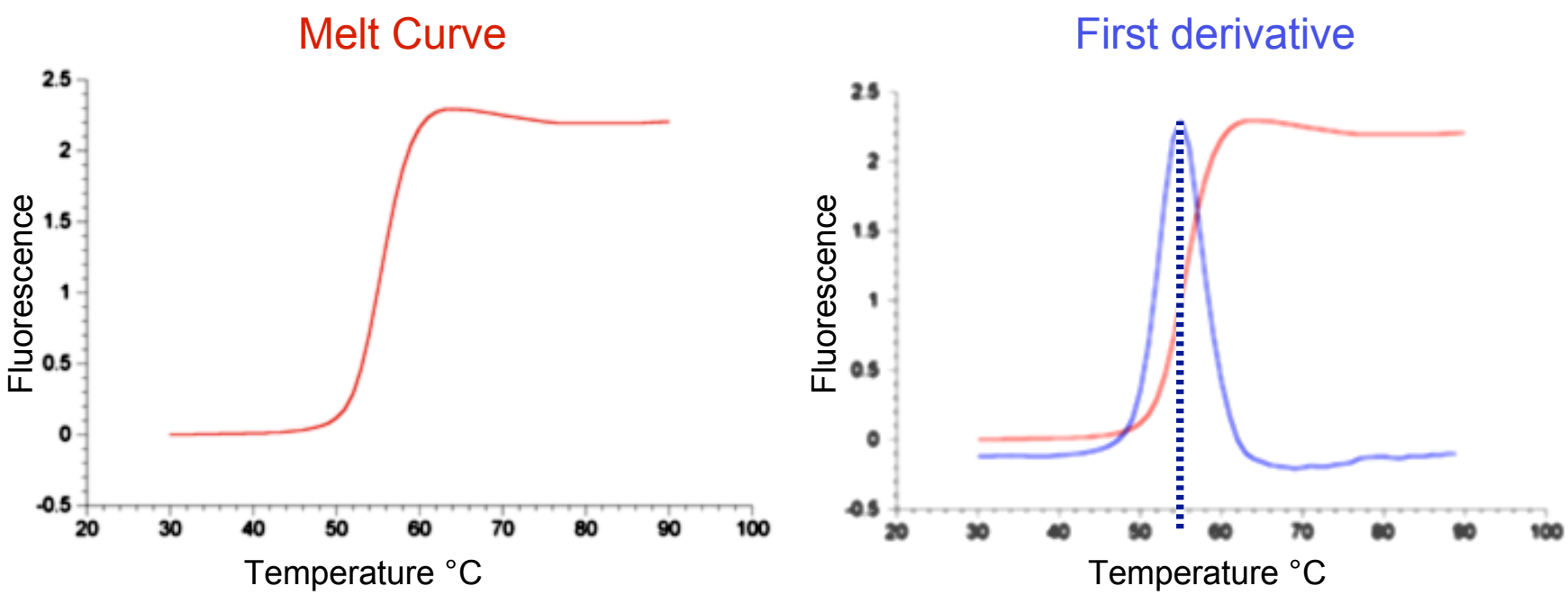
Increasing fluorescence

Methodology

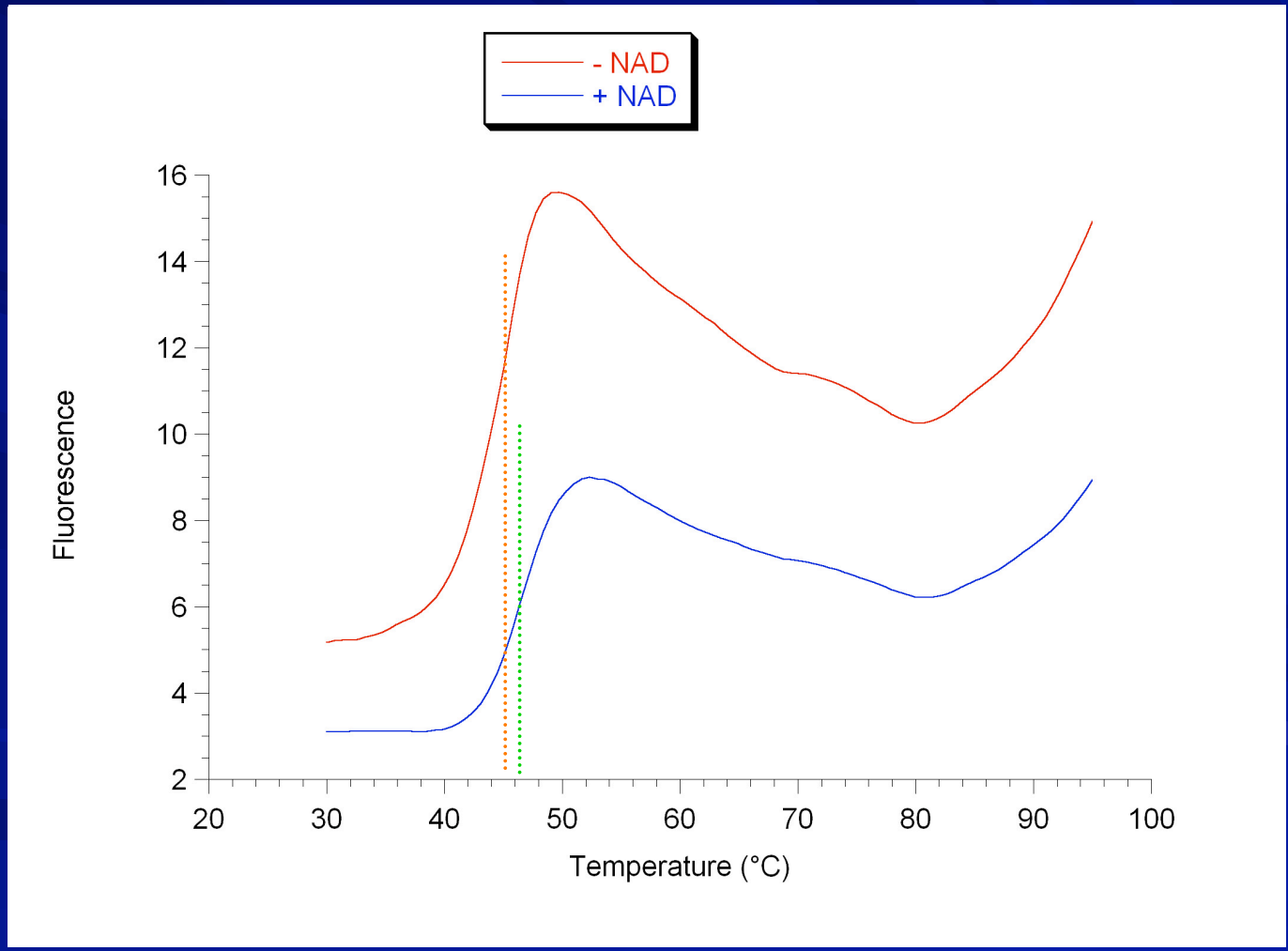
- Assay volume is 15-50 µl with typically 0.1 mg/ml protein (1.5 µg protein per well)
- Use of SYPRO Orange dye allows detection using filters present in a standard RT-PCR machine (Excitation at 473nm and emission at 570nm)
- Each reaction contains only protein and dye – drugs or ligands of interest are added as required
- Heat sample plate from 20 to 90˚C in 0.2 ˚C steps, holding for 12 seconds to read fluorescence



Melt Curves

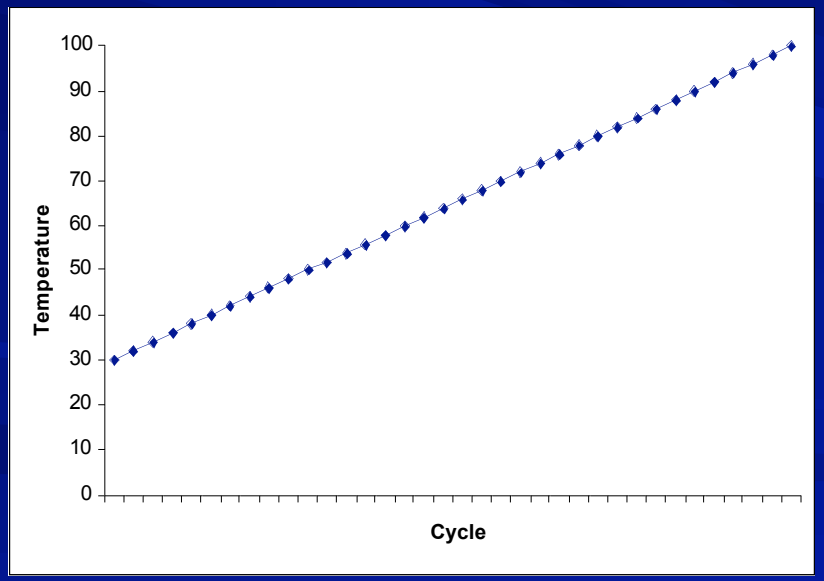

Melt Curve
Fluorescence
2.5
2
1.5
1
0.5
0
-0.5
20
30
40
50
60
70
80
90
100
Temperature °C
First derivative
Fluorescence
2.5
2
1.5
1
0.5
0
-0.5
20
30
40
50
60
70
80
90
100
Temperature °C

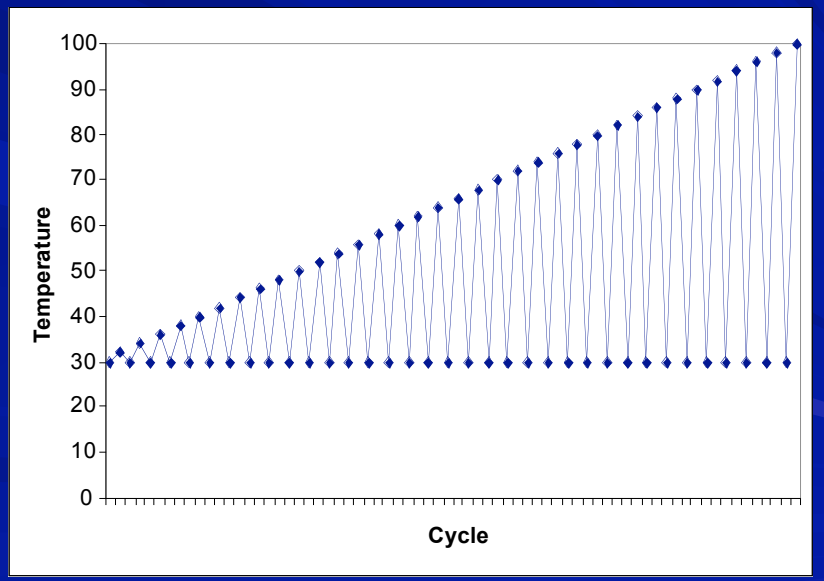
Protein Stabilisation



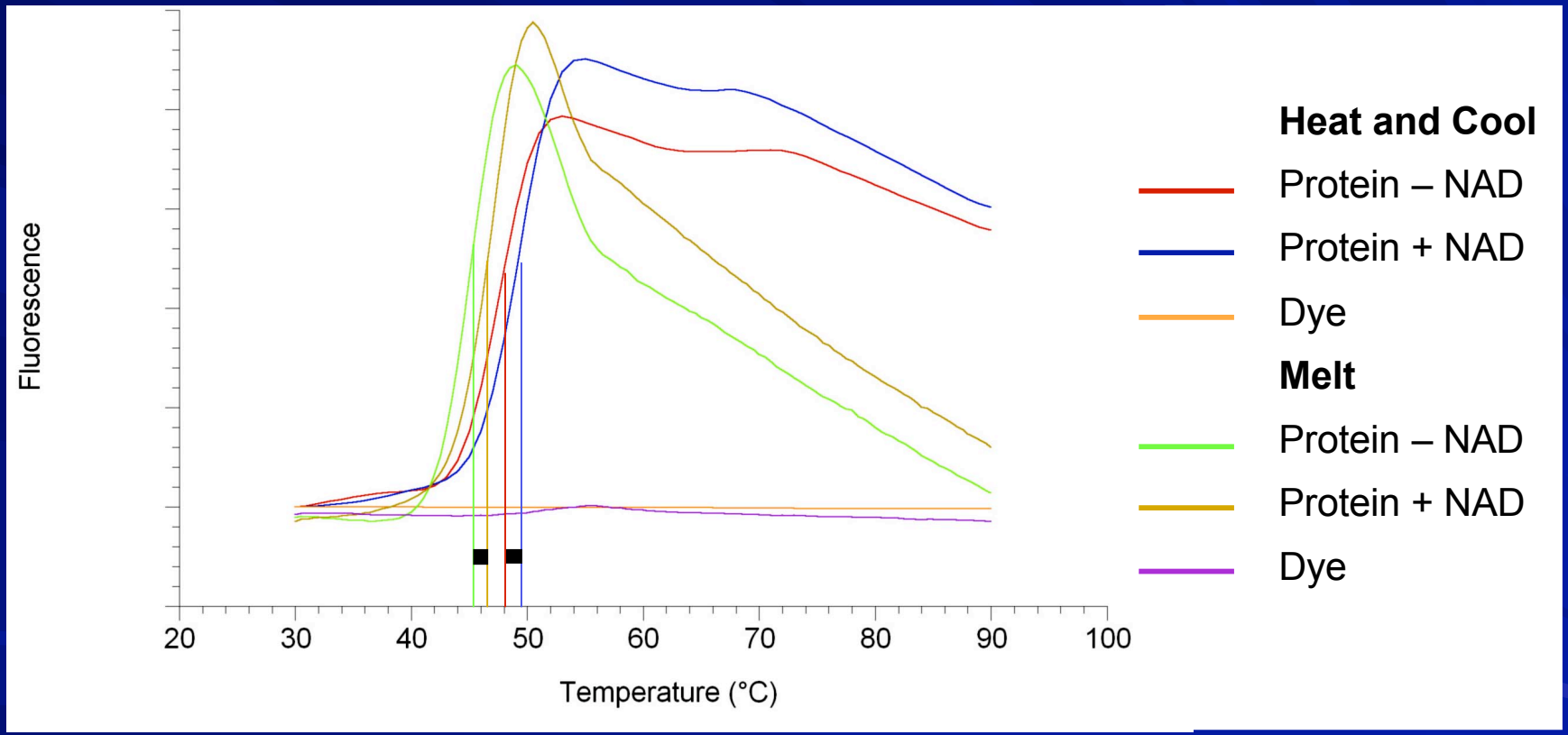
Temperature Cycling Profiles

- Melt
  - fluorescence read at various temperatures
- Heat and Cool
  - fluorescence always read at same temperature
  - takes longer than a melt





Comparison of ‘Heat and Cool’ with ‘Melt’



The change in Tm is the same for “heat and cool” and “melt”

Equipment in the OPPF

- BioRad Opticon 2 Real-Time PCR Dectector
- Plates: Standard white thin-walled PCR plates
- Seals: BioRad Microseal
  - optically clear, adhesive seals

Design and Use of a General Screen

Design of a General Screen

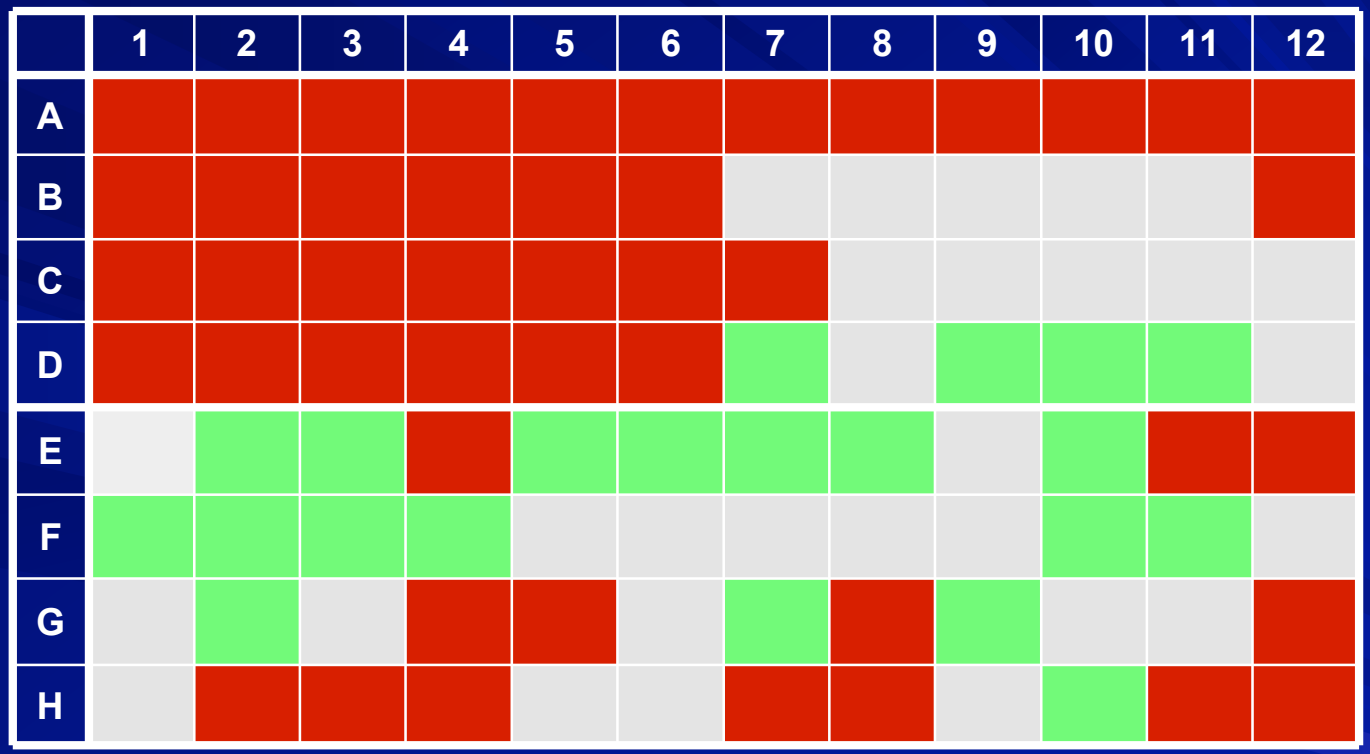
- 96-well PCR plate format
- Contains common changes in buffer condition – pH, type of buffer, salts, etc.
  - Used to find stable buffer conditions for unstable proteins
- Contains common ligands – NAD, ATP, etc.
  - Used to find stabilising ligands and to give information on the function of “unknown” proteins.
  - Provides information for co-crystallisation experiments.

Design of a General Screen

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NaAc pH4.0	NaAc pH4.4	Citrate pH5.0	Citrate pH5.4	Cacodyl pH6.0	Cacodyl pH6.4	HEPES pH7.0	HEPES pH7.4	TRIS pH8.0	TRIS pH8.4	CAPSO pH 9.0	CAPO pH 9.4	No Salt
B	NaAc pH4.0	NaAc pH4.4	Citrate pH5.0	Citrate pH5.4	Cacodyl pH6.0	Cacodyl pH6.4	HEPES pH7.0	HEPES pH7.4	TRIS pH8.0	TRIS pH8.4	CAPSO pH 9.0	CAPO pH 9.4	100mM NaCl
C	NaAc pH4.0	NaAc pH4.4	Citrate pH5.0	Citrate pH5.4	Cacodyl pH6.0	Cacodyl pH6.4	HEPES pH7.0	HEPES pH7.4	TRIS pH8.0	TRIS pH8.4	CAPSO pH 9.0	CAPO pH 9.4	200mM NaCl
D	NaAc pH4.0	NaAc pH4.4	Citrate pH5.0	Citrate pH5.4	Cacodyl pH6.0	Cacodyl pH6.4	HEPES pH7.0	HEPES pH7.4	TRIS pH8.0	TRIS pH8.4	CAPSO pH 9.0	CAPO pH 9.4	500mM NaCl
E	ATP	ADP	AMP	AMPC CP	AMPc PP	AMP PcP	UTP	GTP	GDP	GTP-γS	TMP	FAD	Additives
F	β-NAD	β–γ Methyl GTP	dCMP	dGMP	ssDNA 7mer	ssDNA 9mer	1% glycerol	5% glycerol	10% glycerol	20% glycerol	1mM DTT	5mM DTT	
G	CaCl	MgCl	MnCl	ZiCl	FeCl	KCl	LiCl	Thyio cyanate	L-Proline	Phenol	DMSO	NiCl	
H	Glycine	Sperm-idine	10mM Urea	PEG 400	D(+)-Glucose	D-Galact ose	Alanine	Methio nine	Serine	Arg inine	n-Octyl-β–D-gluco-pyrano side	n-Dodecyl β-D-malto side	

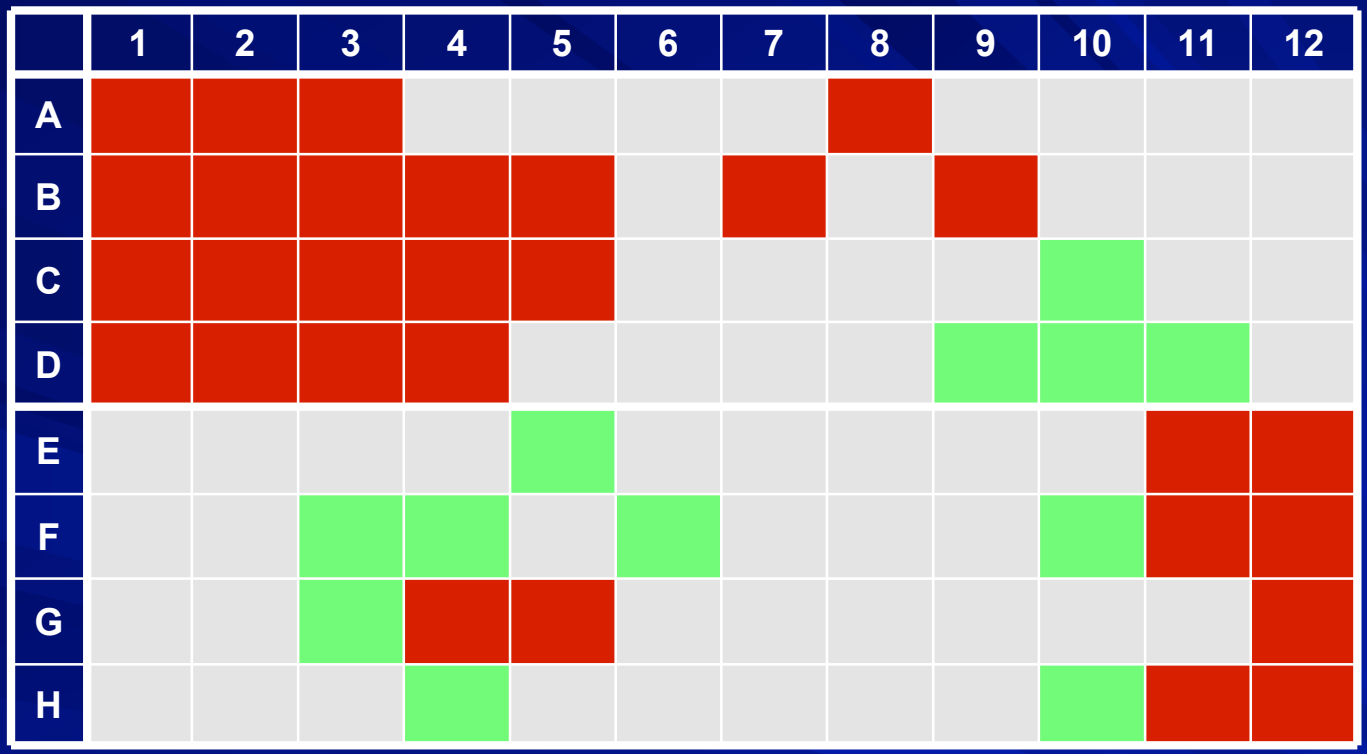
Use of the General Screen

- OPPF2956



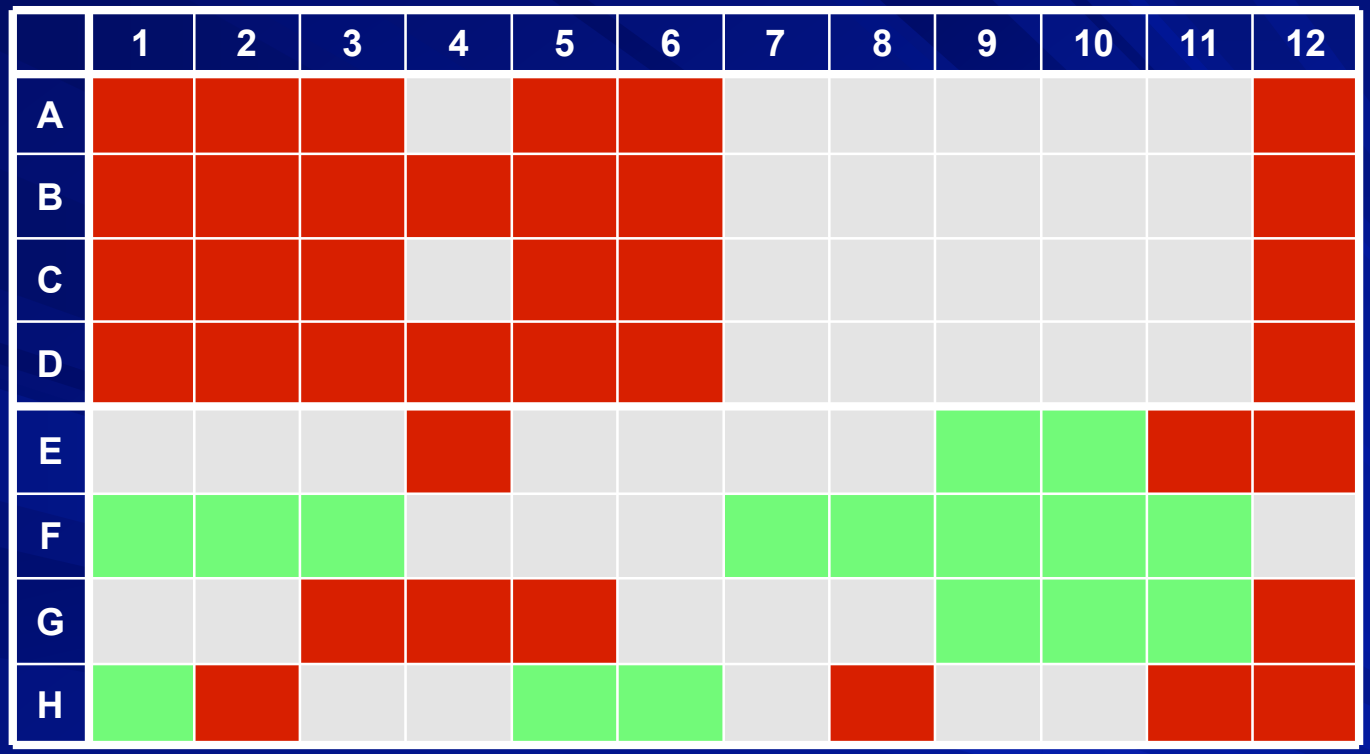
- Protein is more stable in higher salt and pH.
- Protein is stabilised by **nucleotides**, Ca, Mg, proline and arginine.
- OPPF2956 is a DNA binding protein.

- OPPF1446



- Protein is more stable in higher salt and pH.
- Protein is destabilised by **DTT**, **detergent**, Zn, Fe, FAD, TMP.
- OPPF1446 is a dimer which is known to be inactive when dissociated with reducing agents.

- OPPF2398



- Protein is more destabilised outside **pH7-9**.
- **Salt** concentration has no effect on protein stability.
- OPPF2398 is an intracellular domain of a single membrane spanning receptor.

Use in Structural Genomics

- Allows measurement of buffer conditions which favour protein stability:
  - Change of purification buffers and protocol
  - Prevention of protein aggregation
- Screens to find a potential ligands and co-factors:
  - Used in purification to stabilise protein
  - Co-crystallisation trials

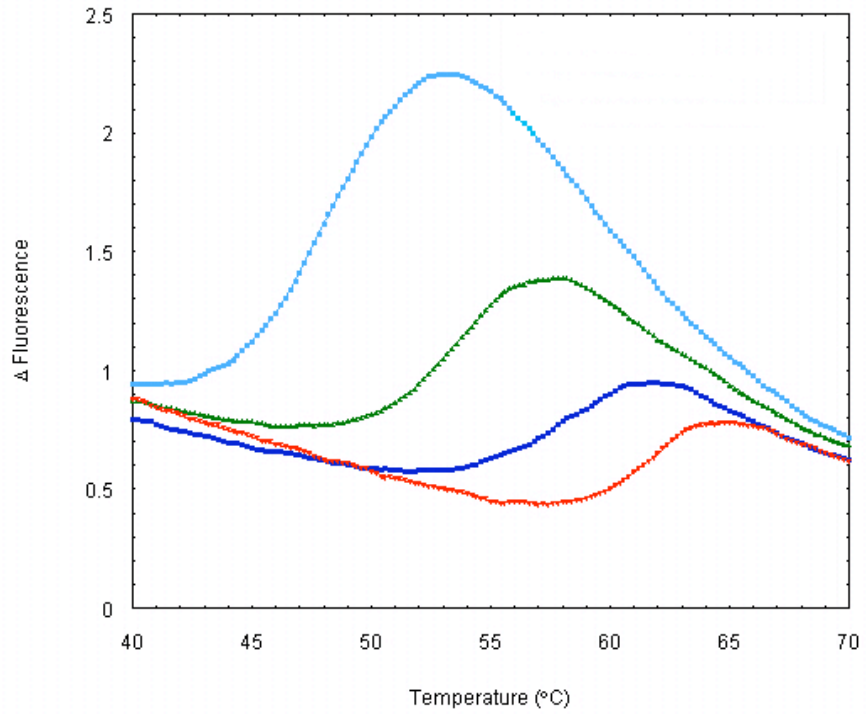
Focussed Ligand-binding Assays

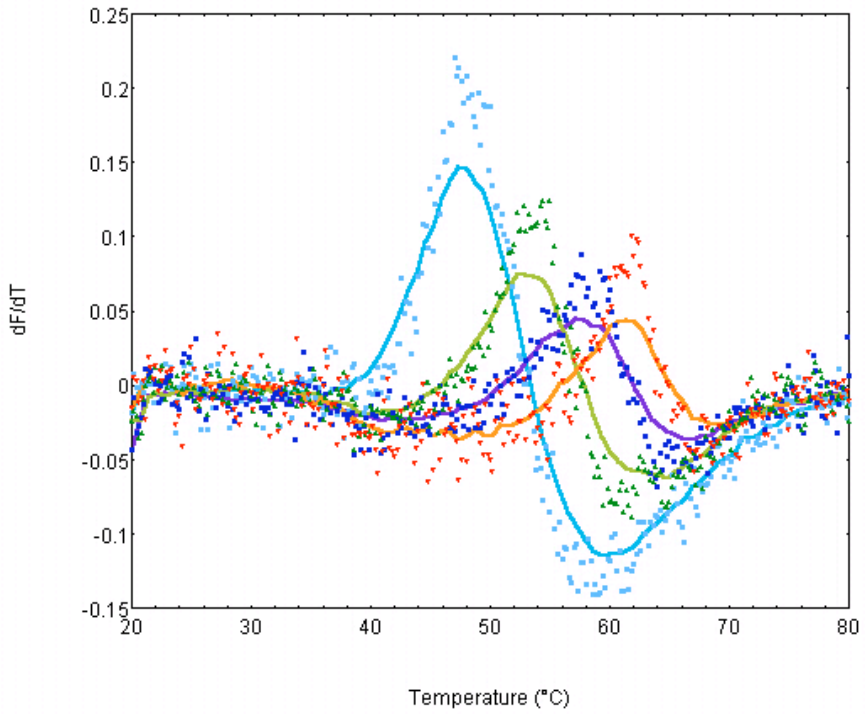
Focussed Sugar-Binding Assay

- C-type lectin

- Protein binds β-glucans
- Structure revealed unpredicted metal binding site
- Test the activity of refolded protein
- Test any metal binding activity

Melt Curve and Derivative Curve





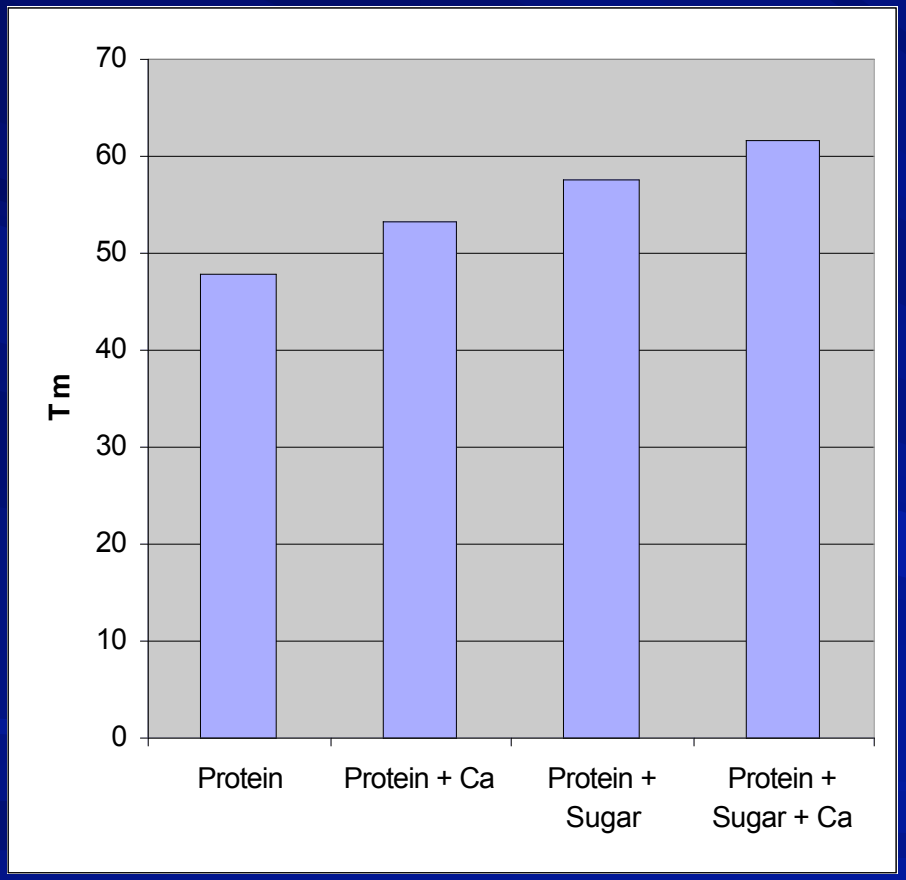
Protein

Protein + Ca

Protein + sugar

Protein + sugar + Ca

Assay Results and Conclusions

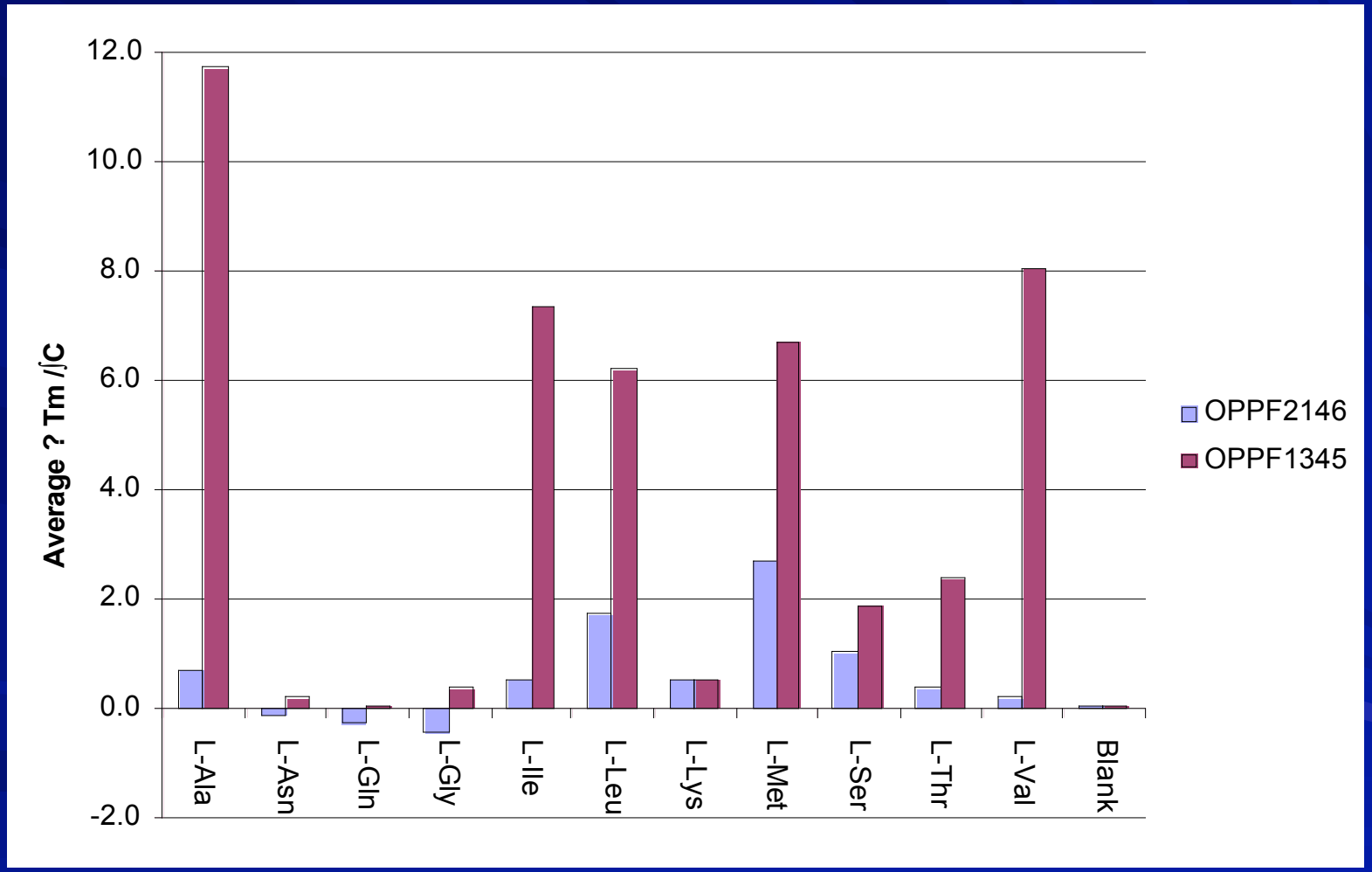


- Sugar binding confirmed by ~10°C shift – confirmed protein correctly refolded
- Metal binding confirmed by ~5°C shift

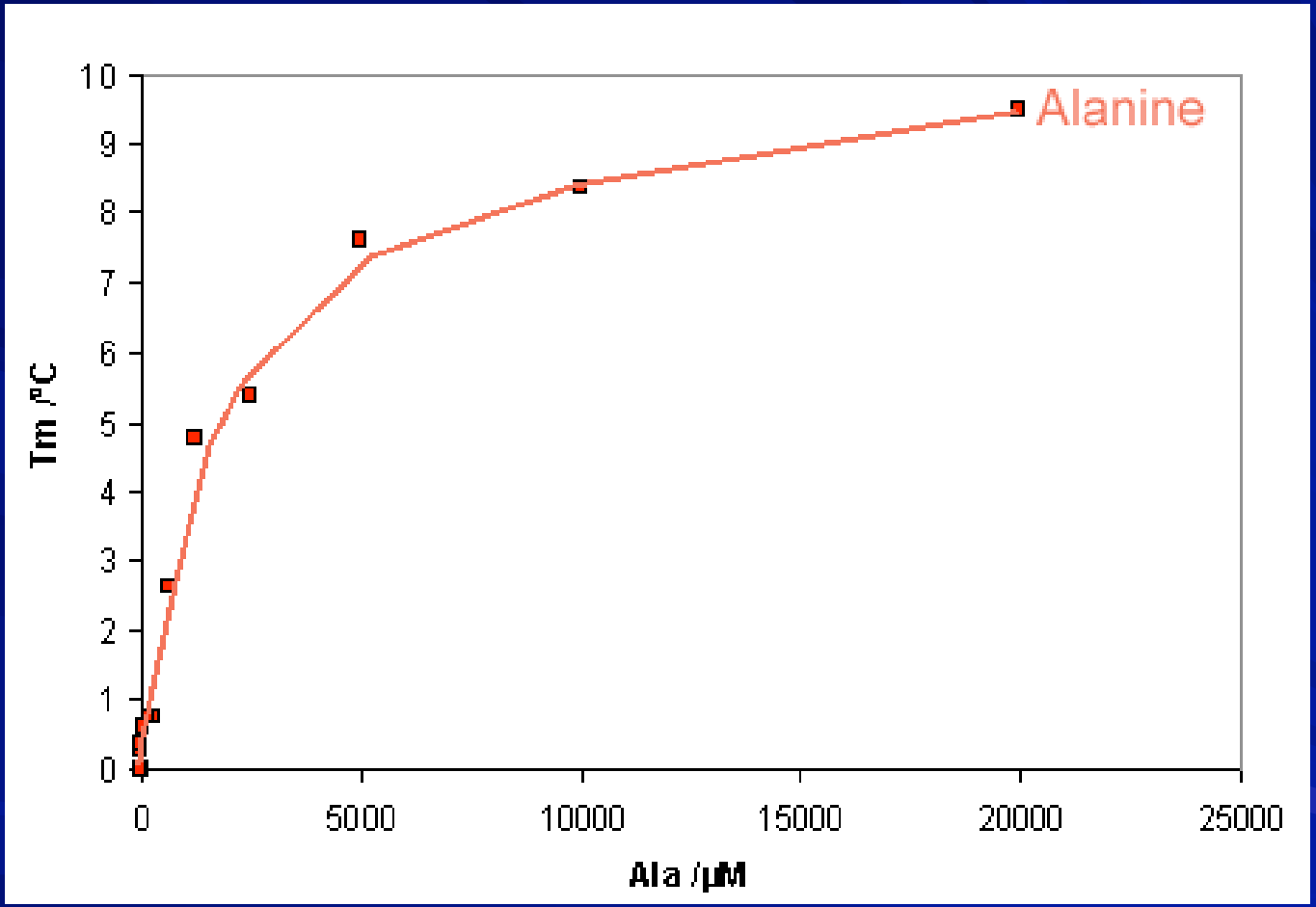
Focussed Amino Acid-Binding Assays

- OPPF1345 and OPPF2146
- Proteins bind amino acids
- *Apo* crystal structures have been solved
- Test the binding of different amino acids
- Use the data for soaking experiments in order to gain crystal structures with co-factors bound

Assay results

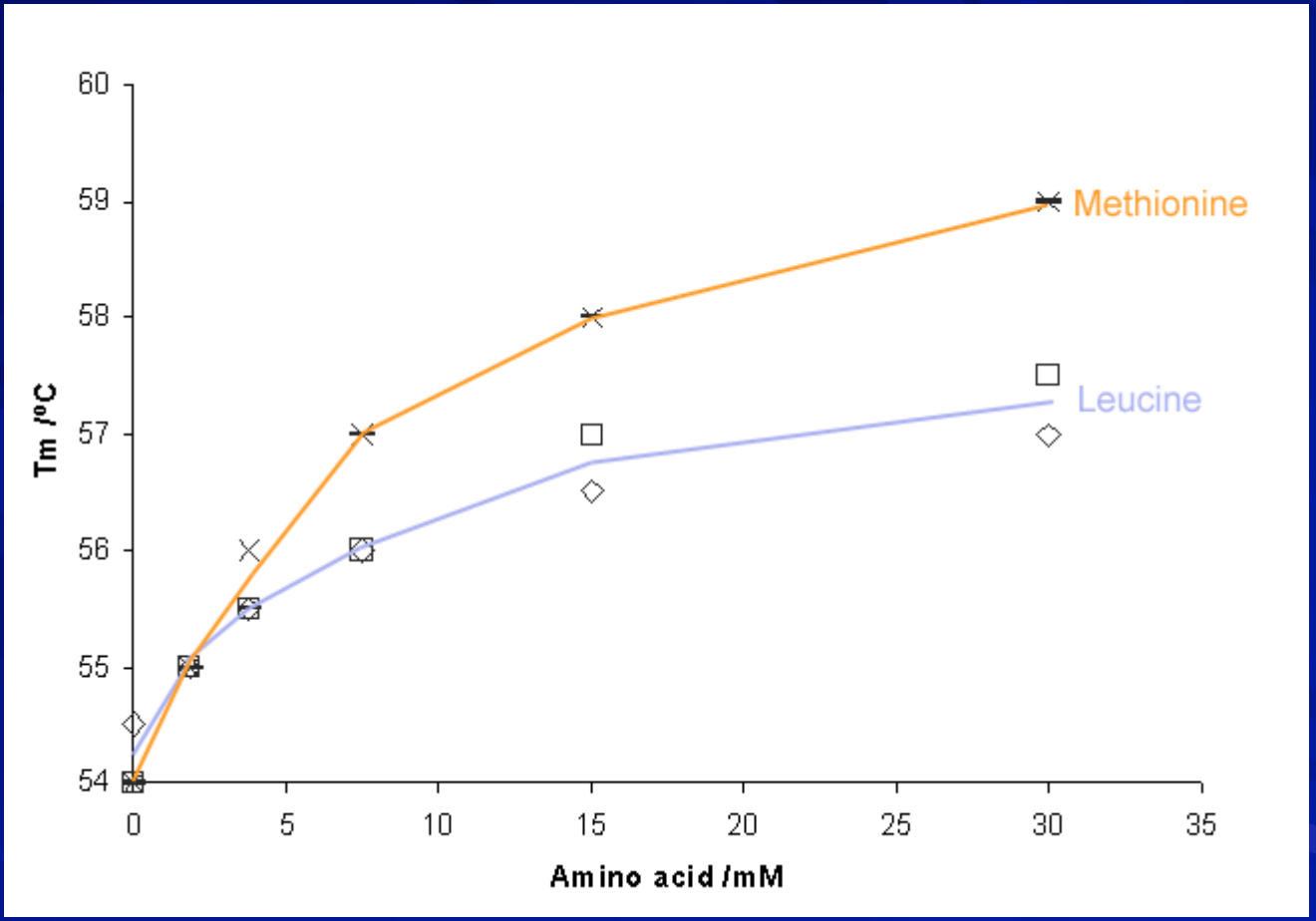


OPPF1345 Alanine Binding



- Co-crystallisation with alanine have been used to obtain a crystals of *holo*-OPPF1345

OPPF2146 Amino-Acid Binding



- Soaks with methionine and leucine have been used to obtain a crystal structures of the protein with amino acids bound

Use in Structural Genomics

- Allows quantification of known ligand binding
- Allows exact ligand to be ascertained eg. NAD^+/NADH/$NADP^+$/NADPH
- Gives information for crystal soaks or co-crystallisation experiments

Limitations

- Some proteins do not give a satisfactory melt curve
- Some co-factors/ligands interact with the dye to give a false positive result
- For a full 96-well screen the technique uses 0.5-1.2mg of protein

References

Methodology:

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Acknowledgements

Methodology:

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General Screen:

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