

# Thermal Shift Assay:

Monitoring solution-dependent changes in protein stability

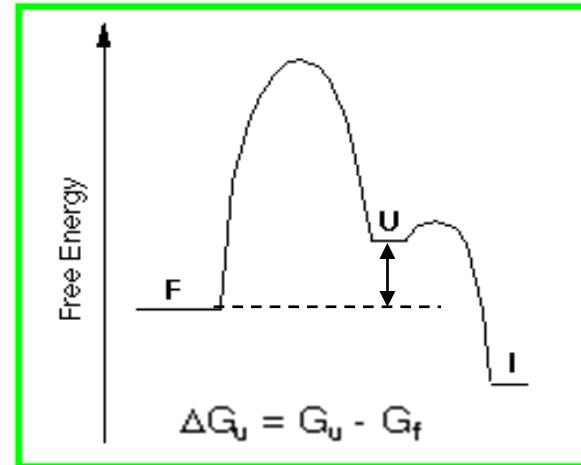
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Department of Biochemistry and Molecular Biology

# Principle:

Thermodynamic stability of a protein is related to its Gibbs free energy of unfolding  $\Delta G_u$ .

For a protein that unfolds and refolds rapidly, reversibly, cooperatively, and with a simple, two-state mechanism

$\Delta G_u$  is simply the difference in Gibbs free energy, G, between the folded and the unfolded states.



**The larger and more positive  $\Delta G_u$ , the more stable is the protein**

The Gibbs free energy, G, is made up of the two terms:  
enthalpy (H) and entropy (S)

$$G = H - TS$$

**At a certain temperature range protein stability is inversely related to temperature.**

$\Delta G_u$  is temperature-dependent:

As the temperature increases, the increase in entropy results in the decrease of the free energy of unfolding:

$$\Delta G_u(T) = \Delta H_u(T) - T\Delta S_u(T)$$

Therefore, for most proteins, as the temperature increases, the  $\Delta G_u$  decreases and becomes zero at equilibrium  $T_m$ , where

$$[\text{folded}] = [\text{unfolded}]$$

**Therefore, protein will be most stable at the conditions where  $T_m$  is the highest**

## Protein stability in solution is influenced by:

- temperature
- pH and proton linkage
- buffer type
- salt type and [ ]
- co-solvents
- metal ions
- preservatives
- surfactants
- excipients
- ligands

## Historically used to test protein stability:

- Differential scanning colorimetry
- circular dichroism
- NMR

## High throughput methods:

•Aggregation: → Differential static light scattering (DSLS)  
(Kurganov, 2002)

•**Tm shift:** → Differential scanning fluorimetry (DSF)  
(Lo, 2004; Ericsson, 2006;  
Vedadi, 2006) aka Protein stability shift assay  
aka Thermal shift assay  
aka Thermofluor™

# Thermal shift assay

## Instrumentation:

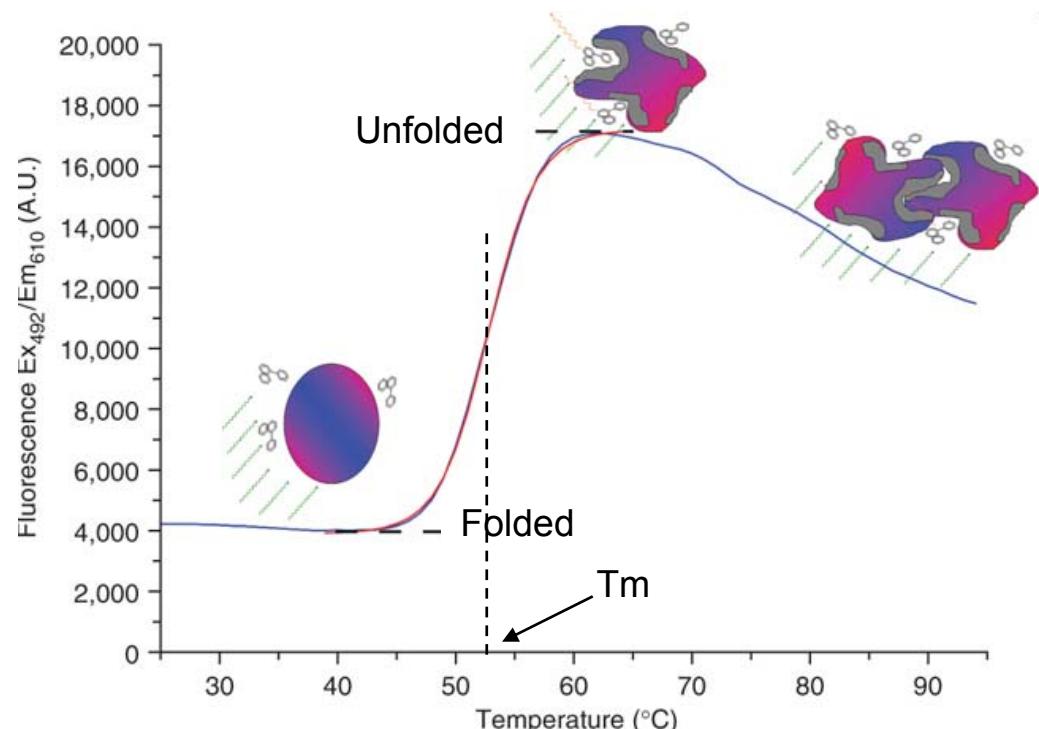
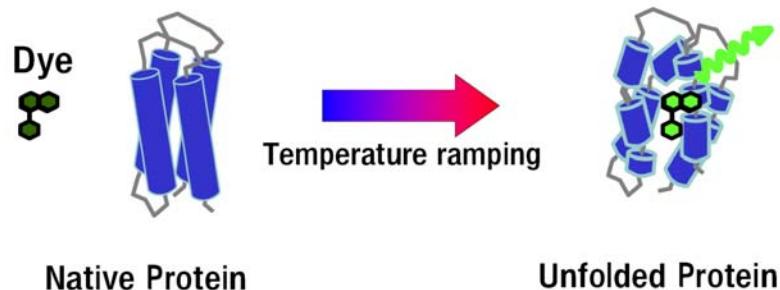
- Fluorescent Plate reader with heated stage, or
- Real time PCR machine

## Reagents:

- Environmentally sensitive fluorescence dye, such as Sypro Orange

## Principle:

- Sypro Orange fluorescence is quenched in an aqueous environment.
- As the temperature rises, the protein undergoes thermal unfolding and exposes its hydrophobic core region.
- Sypro Orange then binds to the hydrophobic regions and becomes unquenched.
- Fluorescence is monitored and plotted versus temperature.
- The midpoint of the protein unfolding transition is defined as the Tm.



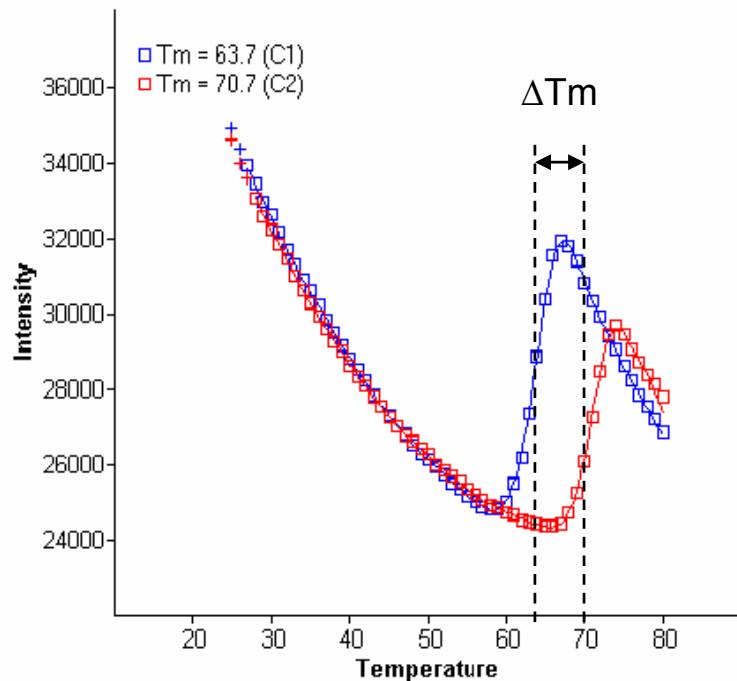
## Applications:

- Condition screening against solutions containing ranges of pH and salt
- Screening against a broad library of physiologically relevant compounds
- Screening against focused libraries for specific proteins and protein families
- Correlation of protein stabilisation and affinity of binding  
(suggested:  $IC_{50} < 1\mu M$  results in  $T_m$  shifts  $> 4^\circ C$  )

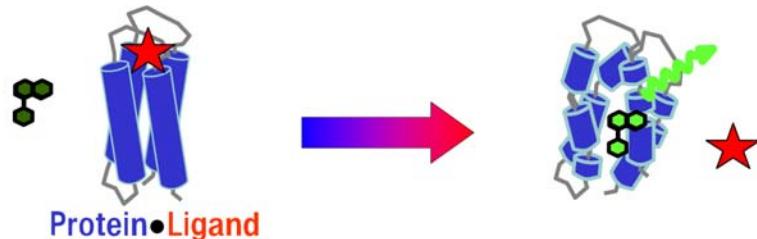
# Condition screening:

The more favorable conditions - the higher Tm

- Changes in buffer composition and pH, salt concentration and presence of compounds that may interact with a native protein will influence the protein Tm, resulting in a temperature shift ( $\Delta T_m$ ).
- In a typical thermal shift experiment, protein and dye containing solution is heated at the rate of 1 degC/min and fluorescence intensity is monitored.
- The melting transition for each condition is presented as fluorescence intensity versus temperature.



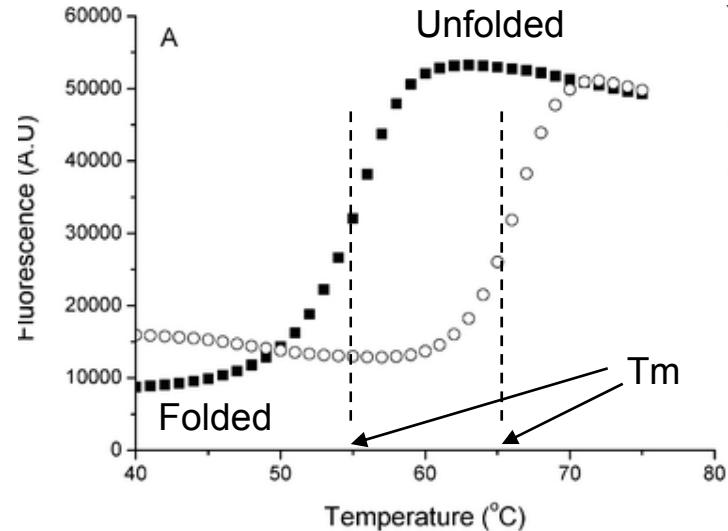
# Ligand screening:



The free energy contribution of ligand binding results in an increase in  $\Delta G_u$ , which frequently causes an increase in  $T_m$ .

The fluorescence-based thermal shift assay is now widely used:

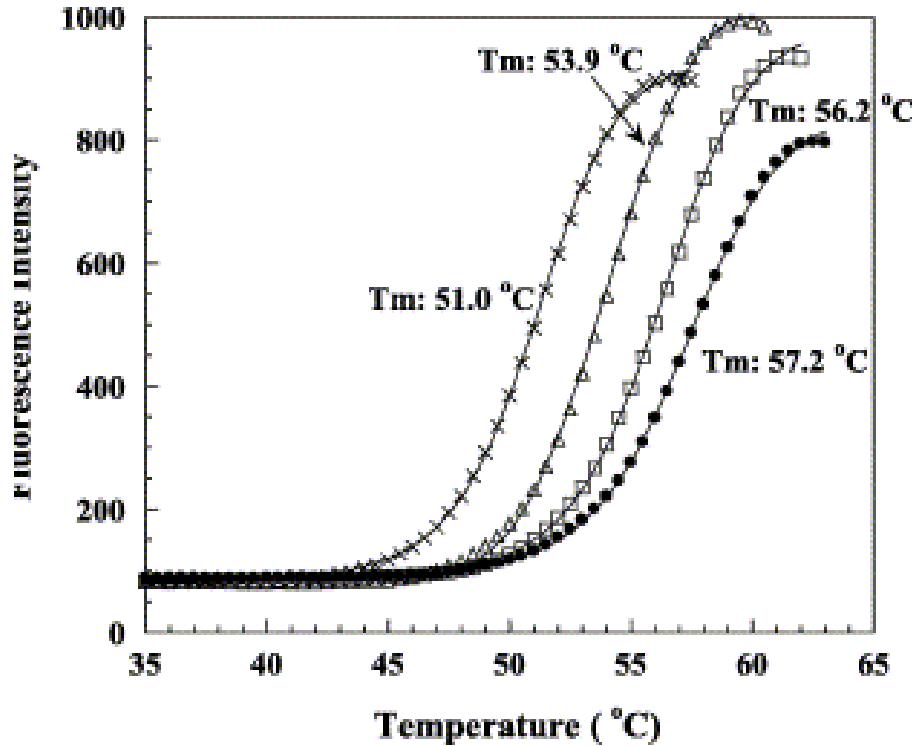
- for study of ligands that can maximise protein stability and minimise aggregation.
- as a method to identify inhibitors of target proteins without even knowing the protein's function and its binding site (structural genomics projects).



Ligand-dependent protein stabilization of the citrate synthase protein by oxaloacetate ligand

# Affinity of ligand binding

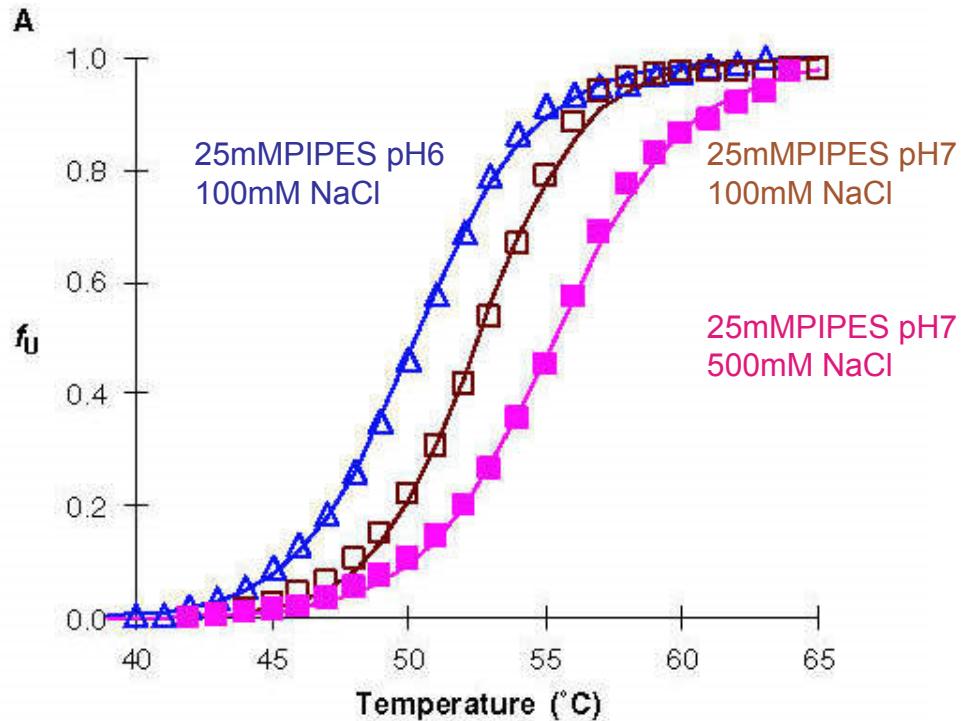
The ligand-binding affinity of any potential inhibitor can be assessed from the shift of the unfolding temperature obtained in the presence vs absence of the potential inhibitor



Unfolding transition of 1  $\mu$ M BACE1 in the presence of 0  $\mu$ M ( $\times$ ),  
10  $\mu$ M ( $\triangle$ ), 50  $\mu$ M ( $\square$ ), and 100  $\mu$ M ( $\bullet$ ) of compound 6

# Protein stability profile

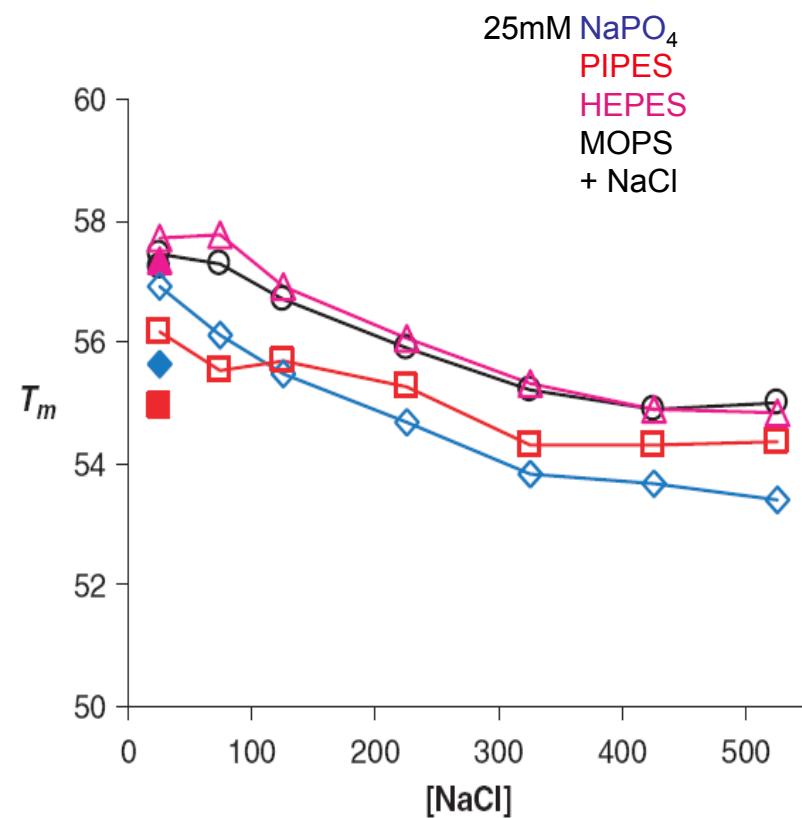
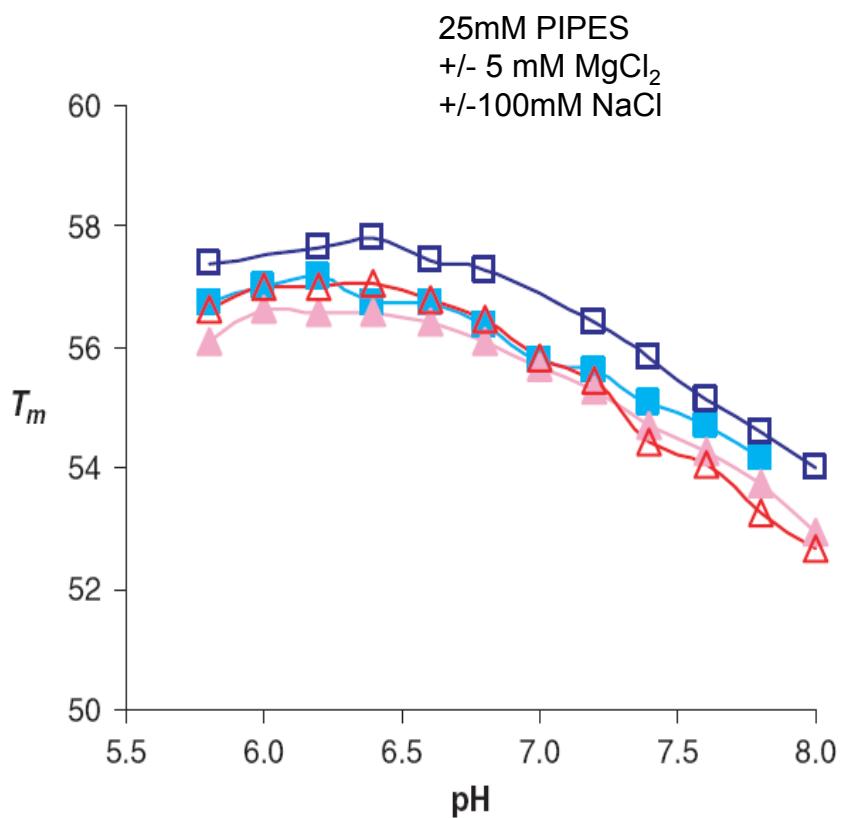
Buffer type and pH, salts, etc...



pKa of buffering compounds

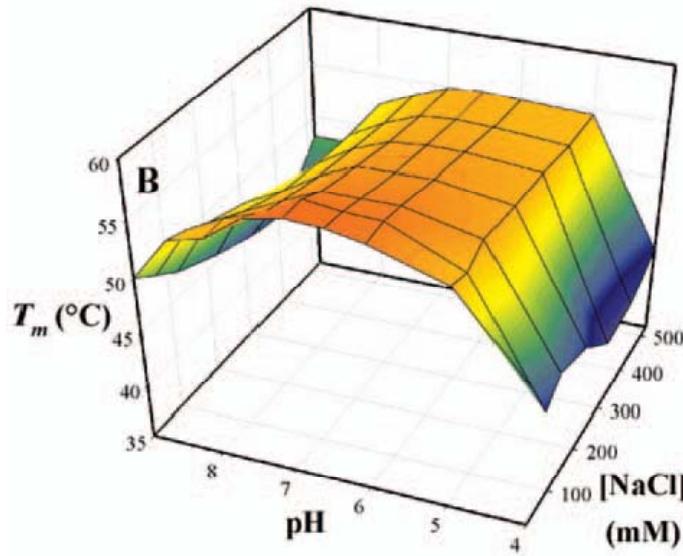
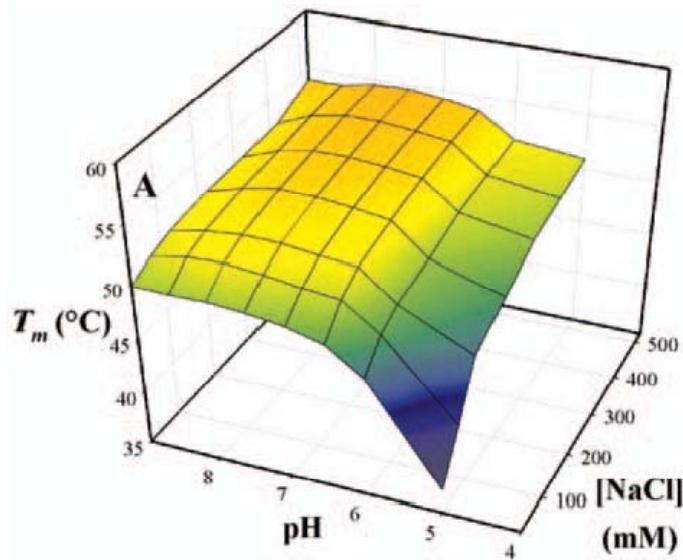
Compound	pKa
Maleate	2.0
Phosphate	2.15
Chloroacetate	2.88
Formate	3.75
Benzoate	4.2
Acetate	4.76
Propionate	4.86
Pyridyne	5.23
Piperazine	(2) 5.55
MES	6.21
Bis-Tris	6.46
ADA	6.62
PIPES	7.1
ACES	6.91
BES	7.26
MOPS	7.31
Phosphate	(2) 7.2
TES	7.61
HEPES	7.66
Tris	8.06
Tricine	8.26
Bicine	8.46
TAPS	8.51
Ethanolamine	9.5
CHES	9.41
CHAPS	10.51
Methylamine	10.62
Piperidine	11.12
Phosphate	(3) 12.33

## Stability ( $T_m$ ) vs pH, ionic strength and buffer



## Protein stability surface:

in this case pH vs [salt]



Buffer	pH
Acetate	4
Acetate	5
MES	6
MES	6.5
HEPES	7
HEPES	7.5
HEPES	8
Borate	8.5

Protein stability surfaces.

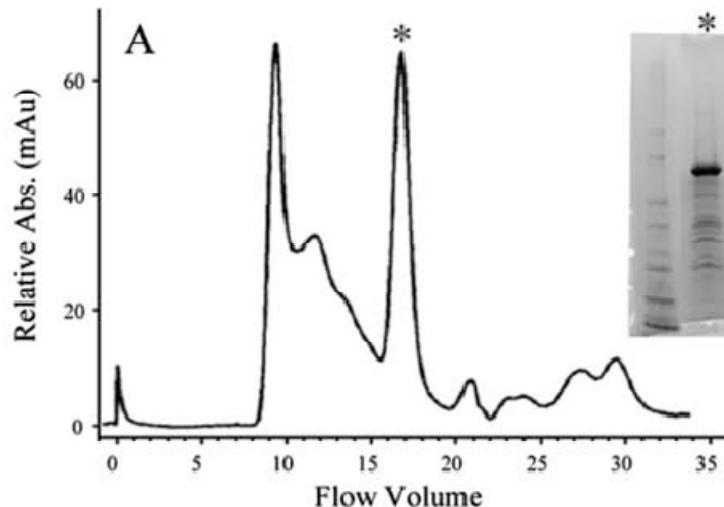
$T_m$  values from the pH-salt profile plotted as a function of NaCl and pH, generating a stability surface for (A) cFMS and (B) Akt-3.

Stability surfaces represent 9 buffers (acetate, pH 4 and 5; MES, pH 6 and 6.5, HEPES, pH 7, 7.5, and 8; borate, pH 8.5; each at 25 mM) and 7 [NaCl] (25, 50, 100, 200, 300, 400, and 500 mM).

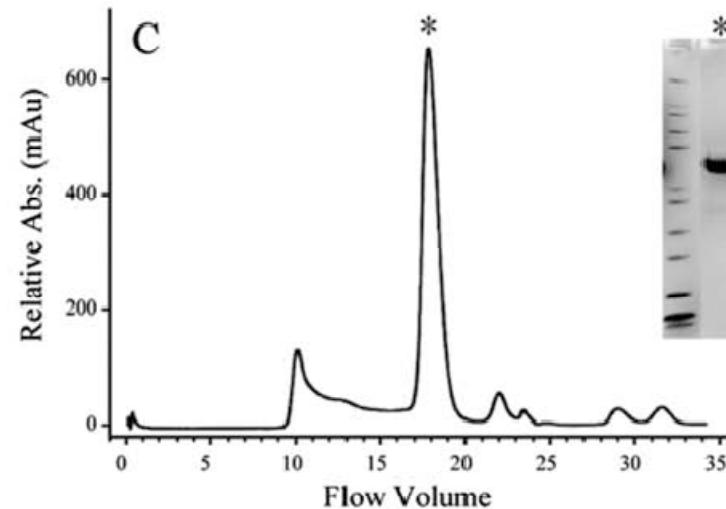
# Optimisation of protein purification protocols

Protein aggregation and purity before and after protein stability profiling - optimised conditions

Before ☹



After ☺



## Original conditions:

- # Tris Buffer, typical salt & reductant,
- GST-column purification
- # Aggregation was biggest challenge

## Altered Purification:

- # Changed to HEPES Buffer
- # Added 10% Glycerol to thrombin cleavage & column elution buffer
- # Minimized Aggregation

## Recommended reading...

### Thermofluor-based stability of proteins

U.B. Ericsson et al. *Anal. Biochem.* 357 (2006) 289–298

### The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability

Frank H Niesen, Helena Berglund & Masoud Vedadi

*Nature Protocols* 2, - 2212 - 2221 (2007)

### Chemical screening methods to identify ligands that promote protein stability, protein crystallization, and structure determination.

M. Vedadi, F. Niesen, A Allali-Hassani, O. Fedorov, P. Finerty, Jr., G. Wasney, R. Yeung, C Arrowsmith, L. Ball, H Berglund, R Hui, B. Marsden, P Nordlund, M Sundstrom, J Weigelt, and A. Edwards  
*PNAS* vol. 103 no. 43 (2006)

### Thermofluor-based high-throughput stability optimization of proteins for structural studies

U.B. Ericsson, B.M. Hallberg, G.T. DeTitta, N.Dekker, P. Nordlund

*Analytical Biochemistry* 357 (2006) 289–298

### Enhancing Recombinant Protein Quality and Yield by Protein Stability Profiling

T. Mezzasalma, J. Kranz, W. Chan, G. Struble, C. Schalk-Hihi, I. Deckman, B. Springer and M. Todd  
*J Biomol Screen* (2007); 12; 418

### High throughput methods of assessing protein stability and aggregation

Guillermo A. Senisterra\* and Patrick J. Finerty, Jr

*Mol. BioSyst.*, 2009, 5, 217–223 | 217

High throughput stability screening for protein crystallisation ...

# Organic Solvents, Additives and Cryoprotectants.

Additive	Concentration	Usage
ethanol	5-20%	solubilization (steroids) additive for crystallization
methanol	5-15%	solubilization (phospholipids combined with MPD)
hexafluoropropanol	1-5%	solubilization (very versatile, peptides and mimetics, steroids, etc.)
2-propanol	5-20%	solubilization(steroids), (cryoprotectant at >70% best in combination with others), additive in crystallization
Glycerol	15-45%	cryoprotectant and additive for crystallization
DMSO	2-20%	solubilization of ligands and cryoprotectant
Ethylene Glycol	15-45%	cryoprotectant
PEG 200-600	35-50%	cryoprotectant, precipitant
Sucrose	>50%w/v	cryoprotectant
MPD	0.5-50%	solubilization (phospholipids combined with methanol) Additive for crystallization, cryoprotectant(25-40%), precipitant
1,6 Hexane diol	0.5-10%	Used instead of MPD as a crystallization additive
1,2,3 Heptane Triol	0.5-15%	Membrane protein crystallization (Amphiphile)
Benzamidine	0.5-15%	Membrane protein crystallization (Amphiphile)
Erythritol	>50%w/v	potential cryoprotectant
Xylitol	20-50%	cryoprotectant when combined with others / compatible with crystallization
Inositol	20-50%	cryoprotectant similar to xylitol
Raffinose	>50%w/v	potential cryoprotectant
Trehalose	20-50%	cryoprotectant when combined with others
Glucose	-	(usually together with other cryoprotectants)
I-2,3-Butanediol	-	cryoprotectant (levo isomer should be best, but racemic mixture also works)
Propylene glycol	-	similar to ethylene glycol
HES hydroxyethyl starch	-	used in cryobiology
PVP polyvinyl pyrrolidone	-	used in cryobiology, potential uses as precipitant

Divalent metals: used for the crystallization of various enzymes, which require such metals for activity. It is important to chose a buffer that will not chelate the metals. Cacodylate, acetate are good candidates. Since metals like zinc, copper cobalt have a tendency to bind to non-specific sites, imidazole chelation may become useful.

Additive	Concentration	Usage
Calcium chloride or acetate	1-20mM	Serine proteases, Avoid phosphoate buffers
Magnesium chloride or sulfate	5-100mM	Can also be used as a precipitant. Avoid Phosphate in the buffer
Zinc acetate or sulfate	0.2-5mM	It reduces protein solubility. It can act as an inhibitor. Essential for activity of some enzymes
Cadmium chloride	0.02-0.2%	Check at concentrations (0.02,0.06,0.12,0.2%). It is very concentration dependent
Barium chloride or acetate	1-20mM	Same as calcium <a href="#">Heavy Atom</a>
Copper sulfate	0.05-2mM	Similar to zinc
Manganese chloride	1-100mM	Calcium or magnesium substitute
Cobalt chloride	1-100mM	Some enzymes depend on it.
Erbium or Samarium acetate	0.1-10mM	use to replace other divalent metals. <a href="#">Heavy Atom</a>

Salts: used to precipitate as well as solubilize proteins. At low concentrations sodium chloride will tend to help increase solubility while at higher concentration will tend to act as a co-precipitant

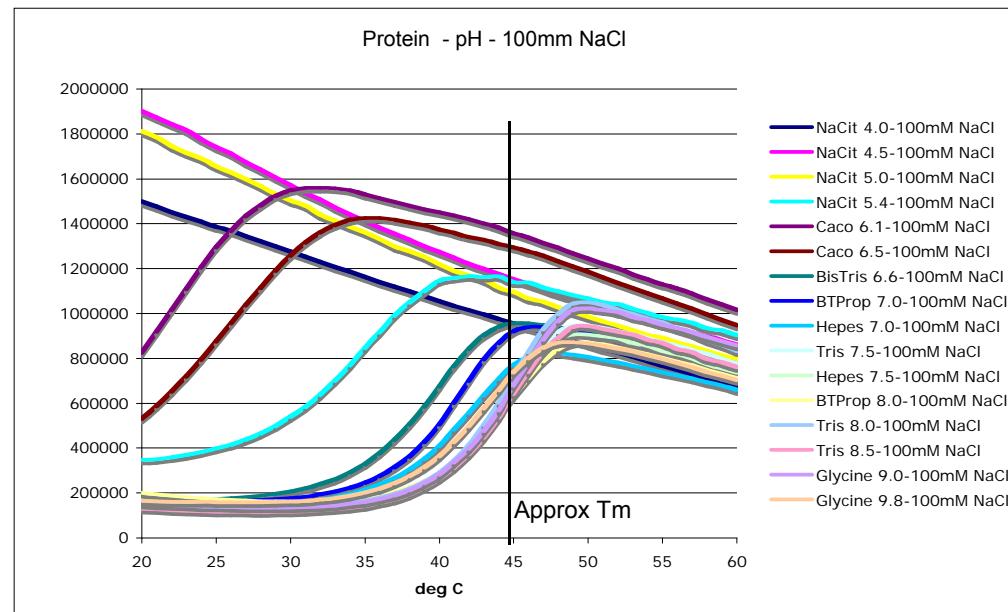
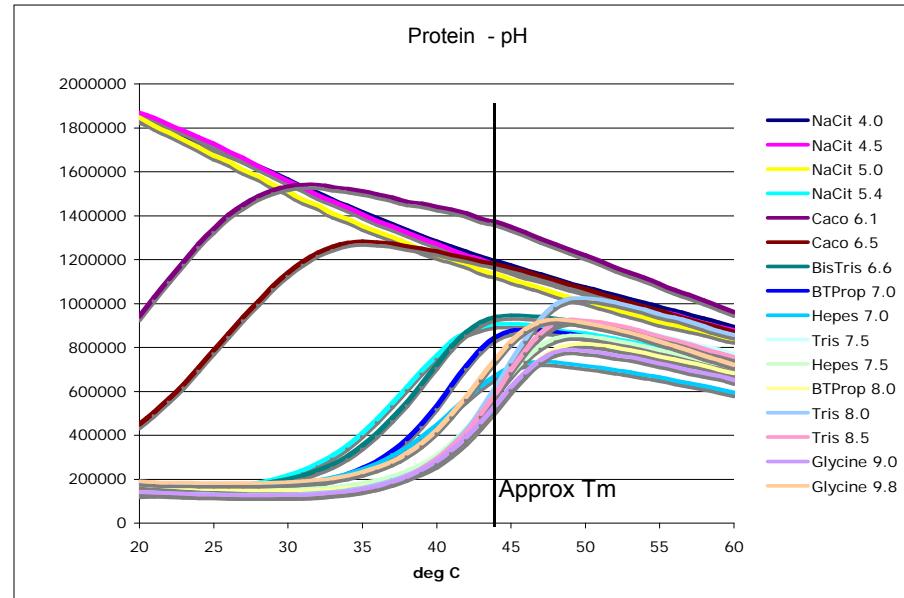
Sodium chloride	50-500mM	Additive to PEG and MPD. May help solubility
Sodium chloride	0.15-2 M	Additive to PEG. Co-precipitant, used to speed up equilibration and gain better control on crystallization with PEG.
Potassium chloride	0.05-2 M	alternative to NaCl. NaCl is generally better
Lithium chloride	0.05-2 M	alternative to NaCl. The differences are worth checking out
Sodium Fluoride	20-300mM	Inhibitor. Use for Divalent metal binding proteins.
Ammonium sulfate (A.S.)	20-300mM	alternative to NaCl as an additive to PEG. Popular in <a href="#">Hampton screens</a>
Lithium sulfate	0.05-2 M	alternative to A.S.
Sodium or Ammonium Thiocyanate	50-500mM	Additive to PEG and MPD and A.S. Acts as a co-precipitant. Of particular interest its use with basic proteins. See references: ( <a href="#">1</a> , <a href="#">2</a> , <a href="#">3</a> )

# Silver Bullets - well composition (example:1/2 plate)

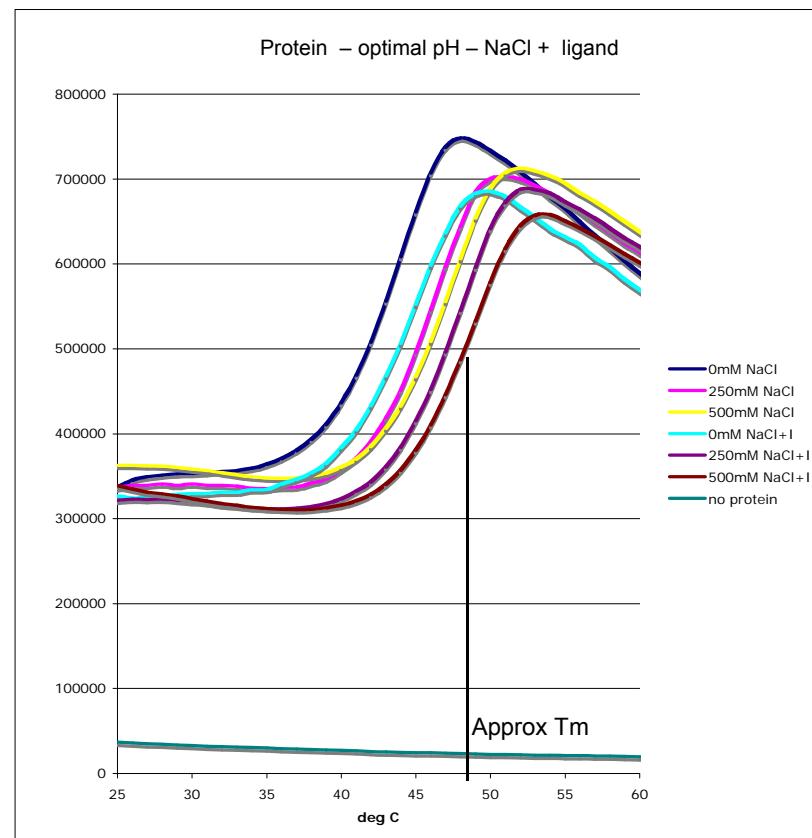
1,5-Naphthalenedisulfonic acid disodium salt	L-Histidine	2,7-Naphthalenedisulfonic acid	Ala-Ala	D-(+)-Maltose monohydrate	Gadolinium(III) chloride hexa	Rhenium(IV) oxide
2,5-Pyridinedicarboxylic acid	L-Isoleucine	Azelaic acid	Ala-gly	D-(+)-Melibiose monohydrate	Samarium(III) chloride hexah	Sodium bromide
3,5-Dinitrosalicylic acid	L-Leucine	trans-Cinnamic acid	Gly-gly-gly-gly	D-(+)-Raffinose pentahydrate	Benzamidine hydrochloride	Sodium nitrate
HEPES sodium pH 6.8	L-Phenylalanine	HEPES sodium pH 6.8	Leu-gly-gly	D-(+)-Trehalose dihydrate	Salicin	Sodium phosphate dibasic dihy
	L-Tryptophan		HEPES sodium pH 6.8	Stachyose hydrate	HEPES sodium pH 6.8	Sodium tetraborate decahydrat
Benzidine	L-Tyrosine	2,6-Naphthalenedisulfonic acid disodium salt		HEPES sodium pH 6.8		HEPES sodium pH 6.8
Nicotinamide	HEPES sodium pH 6.8	2-Aminobenzenesulfonic acid	Aspartame		Calcium chloride dihydrate	
Pyromellitic acid		m-Benzenedisulfonic acid disod	Gly-asp	β-Cyclodextrin	Magnesium chloride hexahyd	Caffeine
Sulfaguanidine	D-(+)-Trehalose dihydrate	HEPES sodium pH 6.8	Gly-ser	D-(+)-Cellobiose	Manganese(II) chloride tetrah	Cytosine
HEPES sodium pH 6.8	Guanidine hydrochloride		Ser-tyr-ZZ	D-(+)-Maltotriose	Zinc chloride	Gallic acid
	Phenol	1,4-Cyclohexanedicarboxylic ac	Tyr-phe	D-(+)-Melezitose hydrate	HEPES sodium pH 6.8	Nicotinamide
Gly-gly	Trimethylamine N-oxide	2,2'-Thiodiglycolic acid	HEPES sodium pH 6.8	D-(+)-Raffinose pentahydrate		Sodium pyrophosphate tetrab
Gly-gly-gly	Urea	5-Sulfoisophthalic acid monosodi	um salt	Stachyose hydrate	Cadmium chloride hydrate	HEPES sodium pH 6.8
Gly-gly-gly-gly	HEPES sodium pH 6.8	HEPES sodium pH 6.8	Ala-alal	HEPES sodium pH 6.8	Cobalt(II) chloride hexahydrat	
Pentaglycine			Aspartame		Copper(II) chloride dihydrate	Digest of Dextran sulfate with
HEPES sodium pH 6.8	2,5-Pyridinedicarboxylic a	3-Aminobenzoic acid	Gly-tyr	Azelaic acid	Nickel(II) chloride hexahydrat	Dextran sulfate sodium salt
	4-Nitrobenzoic acid	3-Aminosalicylic acid	Leu-gly-gly	m-Benzenedisulfonic acid diso	HEPES sodium pH 6.8	Dextranase
3,5-Dinitrosalicylic acid	Mellitic acid	Salicylic acid	L-seryl-L-glutamic acid	Mellitic acid		α-Amylase
4-Aminobenzoic acid	HEPES sodium pH 6.8	HEPES sodium pH 6.8	Tyr-ala	Pimelic acid	3,5-Dinitrosalicylic acid	HEPES sodium pH 6.8
Salicylic acid			HEPES sodium pH 6.8	Pyromellitic acid	3-Indolebutyric acid	
Trimesic acid	Benzidine	Hexamminecobalt(III) Chloride		trans-Cinnamic acid	Naphthalene-1,3,6-trisulfonic	Tryptone
HEPES sodium pH 6.8	Phenylglyoxal monohydrat	Salicylamide	Gly-phe	HEPES sodium pH 6.8	trans-1,2-Cyclohexanedicarbo	HEPES sodium pH 6.8
	Sulfaguanidine	Sulfanilamide	Gly-tyr		HEPES sodium pH 6.8	
4-Nitrobenzoic acid	Sulfanilamide	Vanillic acid	Leu-gly-gly	5-Sulfoisophthalic acid monos	sodium salt	Protamine sulfate
5-Sulfosalicylic acid dihydrate	HEPES sodium pH 6.8	HEPES sodium pH 6.8	HEPES sodium pH 6.8	Anthraquinone-2,6-disulfonic	Betaine anhydrous	HEPES sodium pH 6.8
Naphthalene-1,3,6-trisulfonic acid trisodium salt hydrate				N-(2-acetamido)-2-aminoetha	L-Glutamic acid	
HEPES sodium pH 6.8	Anthrone	p-Coumaric acid	Ala-alal	Tetrahydroxy-1,4-benzoquinon	L-Proline	Digest of Ribonucleic acid an
	Congo Red	Phenylurea	Gly-asp	HEPES sodium pH 6.8	Taurine	Deoxyribonuclease I
2,6-Naphthalenedisulfonic acid disodium salt	N-(2-Acetamido)-2-amino	Poly(3-hydroxybutyric acid)	Gly-gly		Trimethylamine N-oxide dihy	Deoxyribonucleic acid
2,7-Naphthalenedisulfonic acid disodium salt	HEPES sodium pH 6.8	Sulfaguanidine	Gly-phe	1,3,5-Pentanetricarboxylic acid	HEPES sodium pH 6.8	Ribonuclease A
Antraquinone-2,6-disulfonic acid disodium salt			HEPES sodium pH 6.8	5-Sulfosalicylic acid dihydrate		Ribonucleic acid
HEPES sodium pH 6.8	1,3,5-Pentanetricarboxylic acid		Ser-tyr-ZZ	o-Sulfobenzoic acid monoamn	1,2-Diaminocyclohexane sulf	HEPES sodium pH 6.8
	5-Sulfosalicylic acid dihyd	1,2-Diaminocyclohexane sulfate	HEPES sodium pH 6.8	Sodium 4-aminosalicylate dihy	4-Nitrobenzoic acid	
1,5-Naphthalenedisulfonic acid disodium salt	Trimesic acid	1,4-Cyclohexanedicarboxylic acid		HEPES sodium pH 6.8	Cystamine dihydrochloride	Digest of Casein and Hemogl
Naphthalene-1,3,6-trisulfonic acid trisodium salt hydrat	HEPES sodium pH 6.8	Methylenediphosphonic acid	Glycine		Spermine	Casein
PIPER		Sulfanilic acid	L-(+)-Threonine	CHAPS	HEPES sodium pH 6.8	Hemoglobin
HEPES sodium pH 6.8	5-Sulfoisophthalic acid mo	HEPES sodium pH 6.8	L-(+)-Lysine	HEPES		Pepsin
	Cystathione		L-Alanine	Tris	1,5-Naphthalenedisulfonic ac	Protease
1-Pentanesulfonic acid sodium salt monohydrate	Dithioerythritol	D-Fructose 1,6-diphosphate tris	L-Arginine	Hexamminecobalt(III) Chlorid	2,7-Naphthalenedisulfonic aci	Proteinase K
3,5-Dinitrosalicylic acid	L-Citrulline	D-Glucose 6-phosphate	L-Asparagine monohydrate	HEPES sodium pH 6.8	5-Sulfoisophthalic acid monos	Trypsin
3-Aminosalicylic acid	HEPES sodium pH 6.8	L-O-Phosphoserine	L-Aspartic acid		Sulfanilic acid	HEPES sodium pH 6.8
Salicylamide		O-Phospho-L-tyrosine	L-Glutamic acid	MES monohydrate	HEPES sodium pH 6.8	
HEPES sodium pH 6.8	3,5-Dinitrosalicylic acid	HEPES sodium pH 6.8	L-Glutamine	PIPER		Ovalbumin digested with Prote
	3-Aminobenzenesulfonic acid		L-Histidine	Hexamminecobalt(III) Chlorid	2,6-Naphthalenedisulfonic aci	Ovalbumin
5-Sulfosalicylic acid dihyd	Benzamidine hydrochloride	L-Isoleucine		HEPES sodium pH 6.8	4-Aminobenzoic acid	Pepsin
HEPES sodium pH 6.8	L-Carnitine hydrochloride	L-Leucine			5-Sulfosalicylic acid dihydrat	Proteinase K
		L-Cystine	L-Methionine		Naphthalene-1,3,6-trisulfonic	Trypsin
		L-Ornithine hydrochloride	L-Phenylalanine		HEPES sodium pH 6.8	HEPES sodium pH 6.8
		HEPES sodium pH 6.8	L-Proline			
			L-Serine			
			Caffeine	L-Tryptophan		
			Dithioerythritol	L-Tyrosine		
			L-Methionine	L-Valine		

# Examples

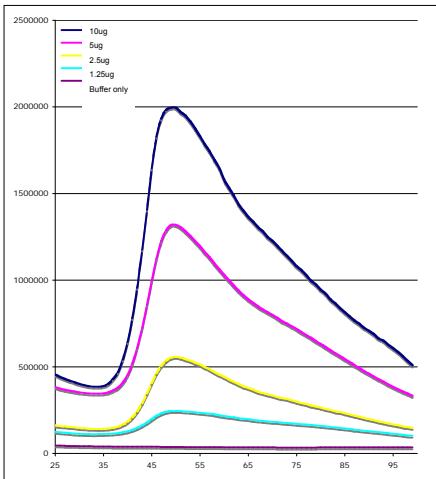
## Condition screening



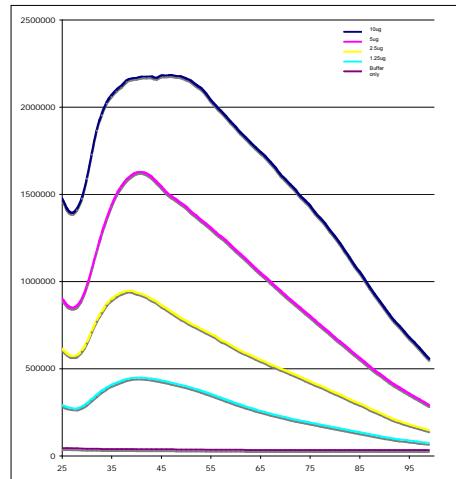
## Ligand screening



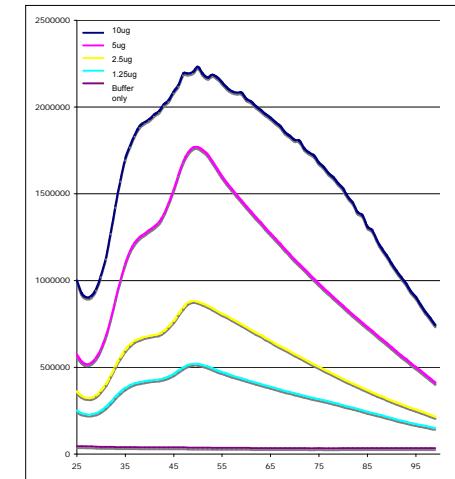
Protein A



Protein B

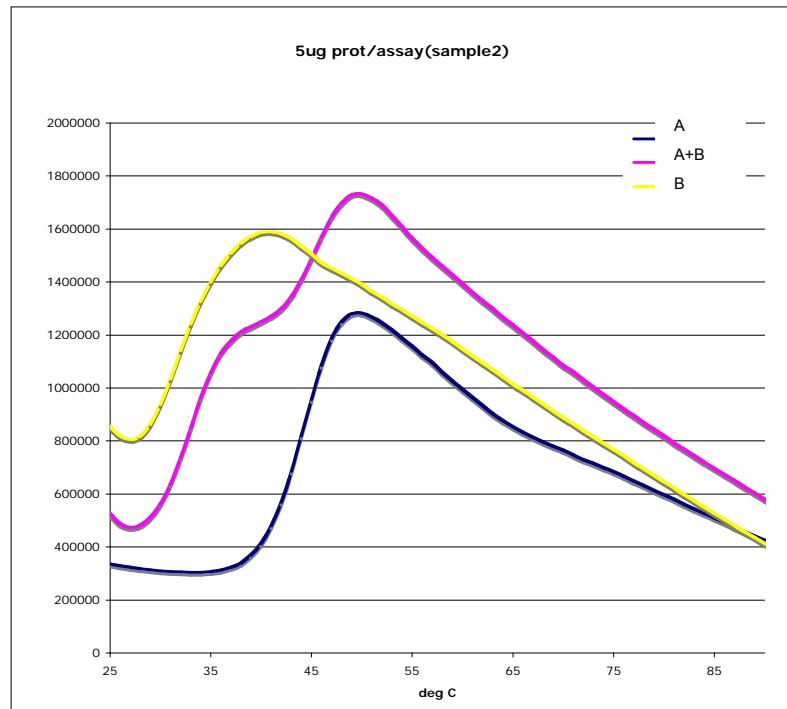


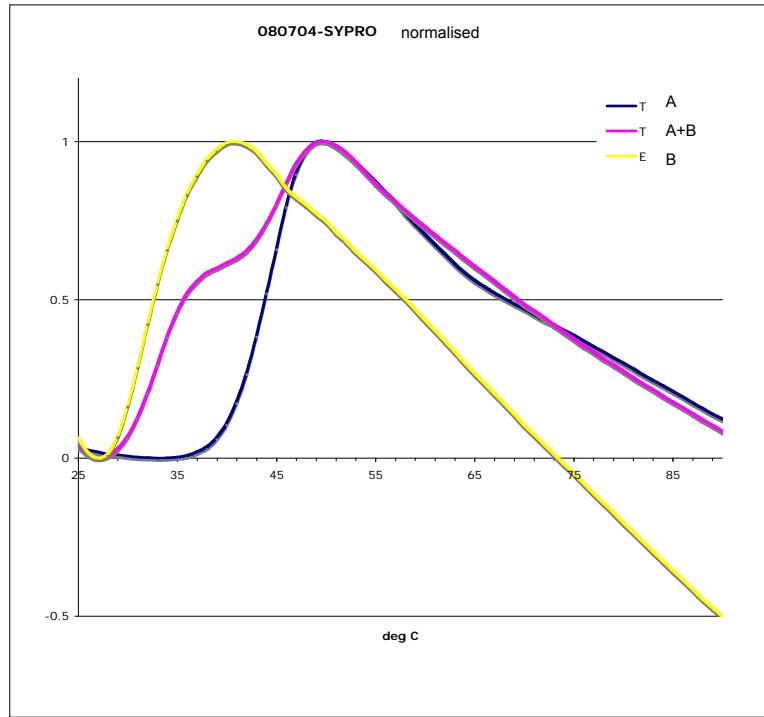
Complex A+B



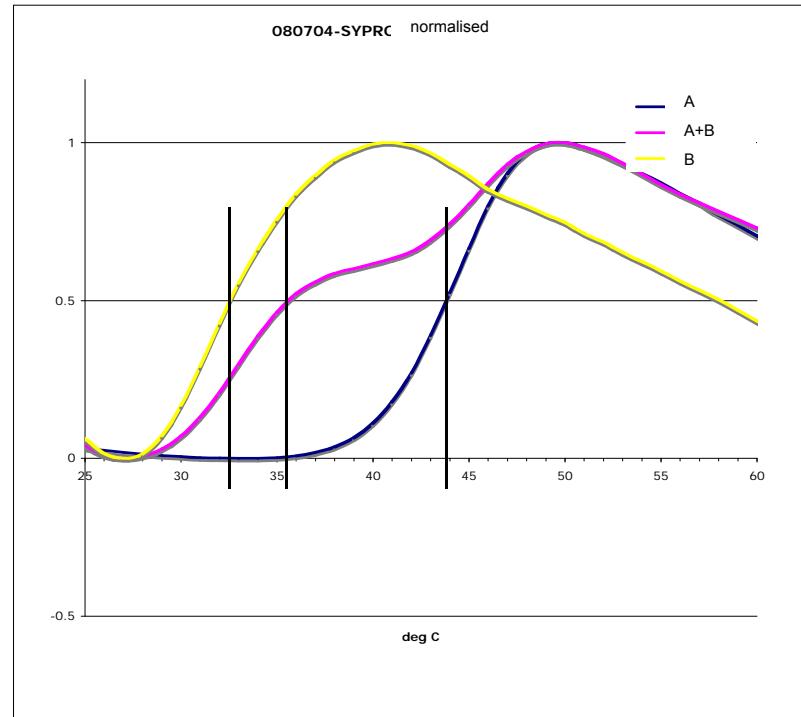
1. Fluorescence intensities for four concentrations of protein were tested by performing a pilot experiment

2. Suitable protein concentration was chosen for subsequent experiments



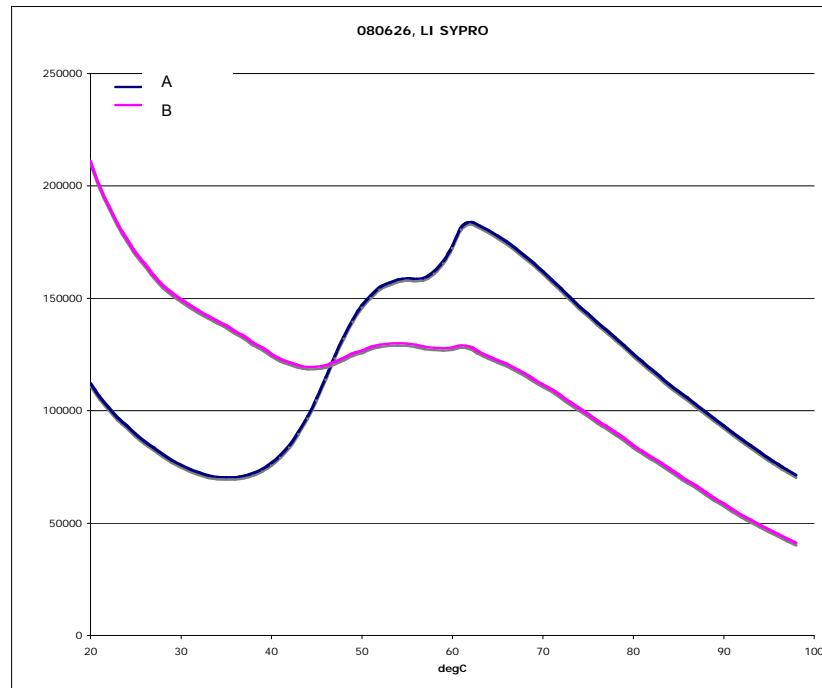


3. Data was normalised



4. Tm was identified from the midpoint of each melt curve

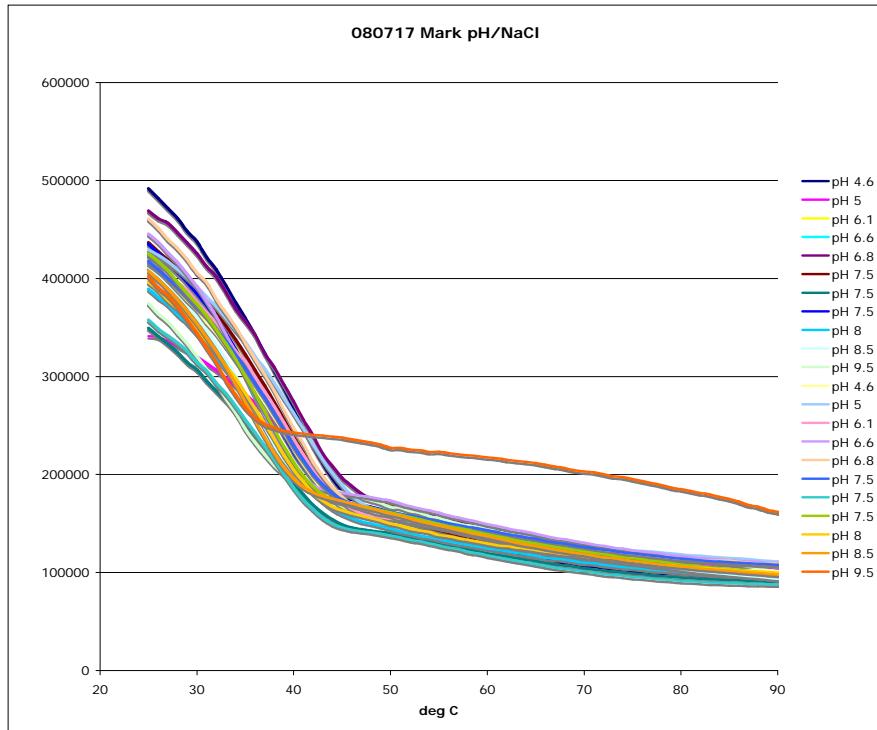
NOTE: The Tm of a protein can be calculated from the inflection point of the melt curve by a simple curve-fitting procedure using an inbuilt data analysis program of the qPCR instrument, but be aware that not all proteins and especially protein complexes are unfolding in a cooperative manner according to a two – state transition.



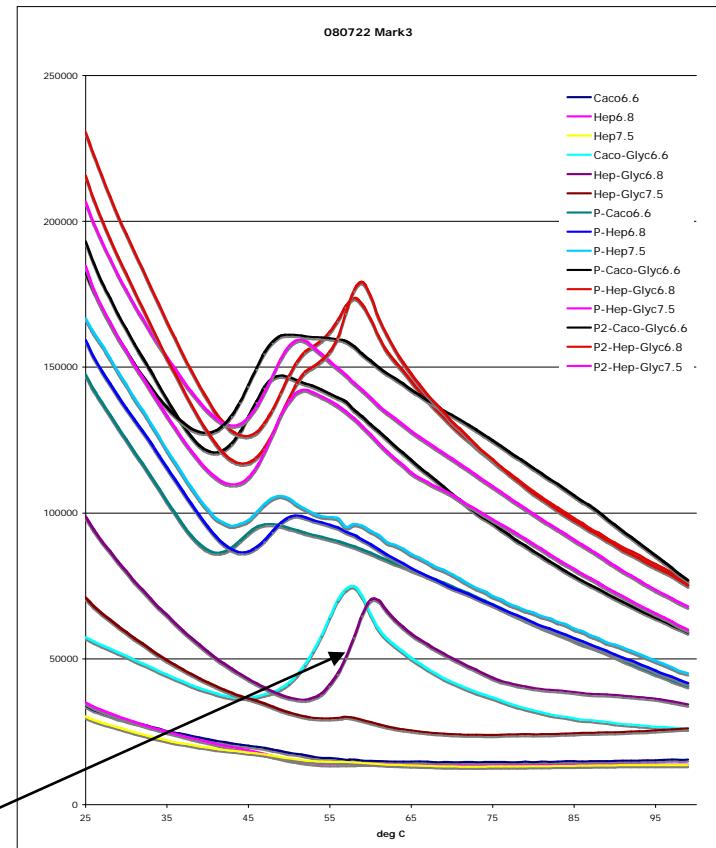
Sample A : protein-peptide complex, biphasic melt curve

Protein B: unstable at the experimental conditions, no typical melt curve

# Optimisation of protein purification protocols



Starting point ☺



Promising.....

# Block temperature uniformity test, Mx3005P

