

Streamlining Assay Development:

Lessons in Process Optimization Through Protein Optimization

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Lead Generation Biology

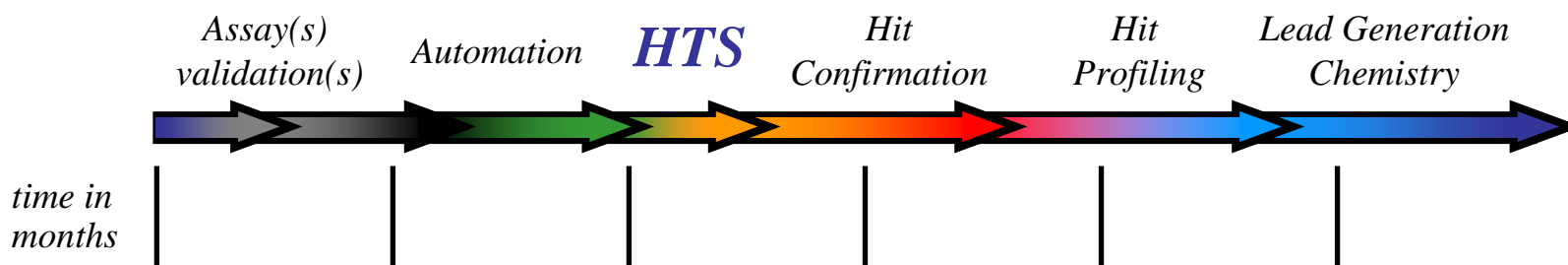
September 22, 2005



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Rate Limiting Steps in Lead Generation

- Industry-wide, HTS (pushing plates) is a small part of the total process in Lead Generation.

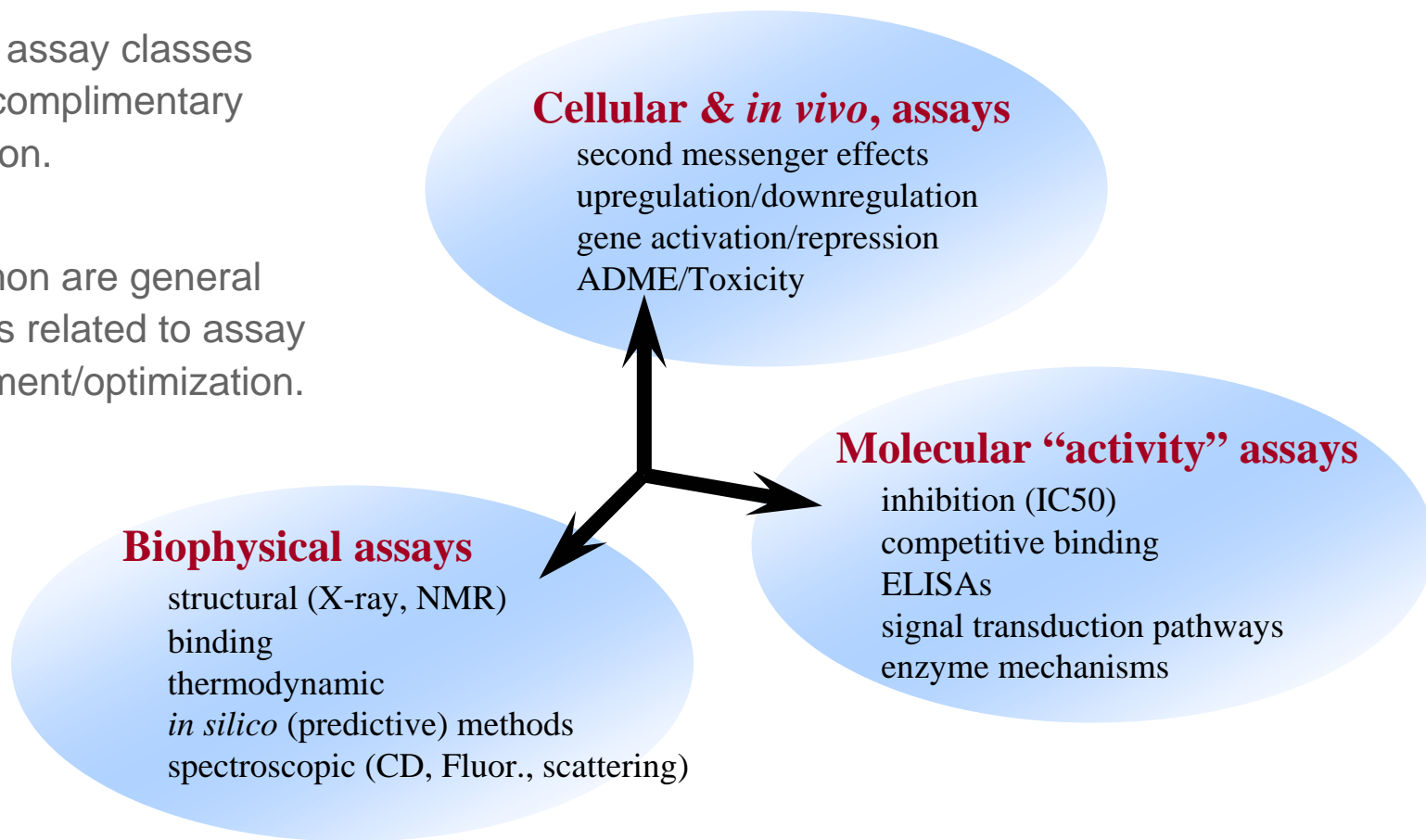


- ✓ Can we streamline the upstream components; assay development, validation, & automation?



Multidimensional Biological Approach

- ❑ Different assay classes provide complimentary information.
- ❑ In Common are general questions related to assay development/optimization.



Problems of Protein Stability

Susceptibility of Proteins to Degradation

Chemical, Covalent Degradation:

- Deamination
- Oxidation
- Disulfide bond shuffling

★ Physical Degradation:

- Protein Unfolding
- Loss through adsorption to Surfaces
- Nonnative Aggregation



Protein Stability by *ThermoFluor*[®]

Factors Influencing Protein Stability

Temperature:

- Parabolic dependence on ΔG (cold and heat denaturation).
- High Temperature can result in **irreversible unfolding**.

Preservatives (formulation):

- Added to ensure sample sterility.
- Can induce aggregation in the absence of additional stabilizers.

Surfactants:

- Added to prevent aggregation and adsorption to surfaces.
- Can destabilize native protein, while kinetically inhibiting aggregation.

★ Salt Type and Concentration:

- Complex effects on protein stability, solubility, and aggregation rates.
- Net effect on protein stability is a balance of multiple mechanisms.

★ Solution pH:

- Determines total charge on a protein.
- **Strong influence** of pH on protein **aggregation rates**.

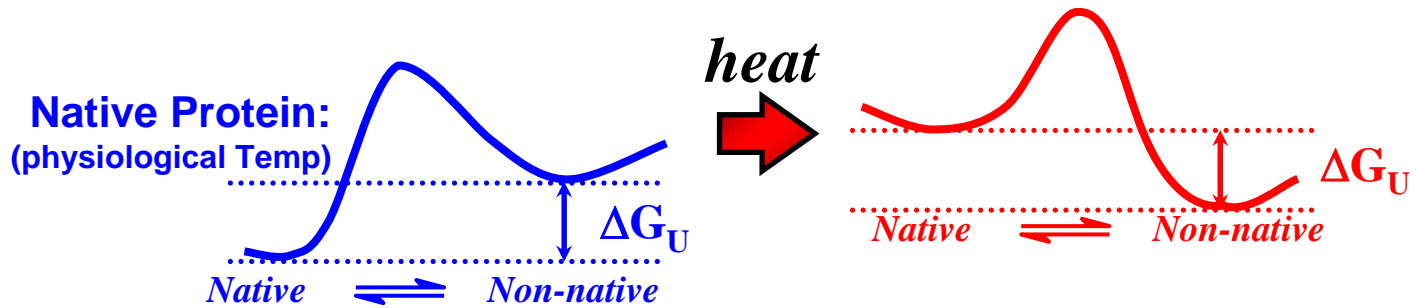
★ Ligands & Cosolutes:

- Compound binding *generally* will stabilize native protein.
- Preferential hydration by cosolutes can prevent unfolding.

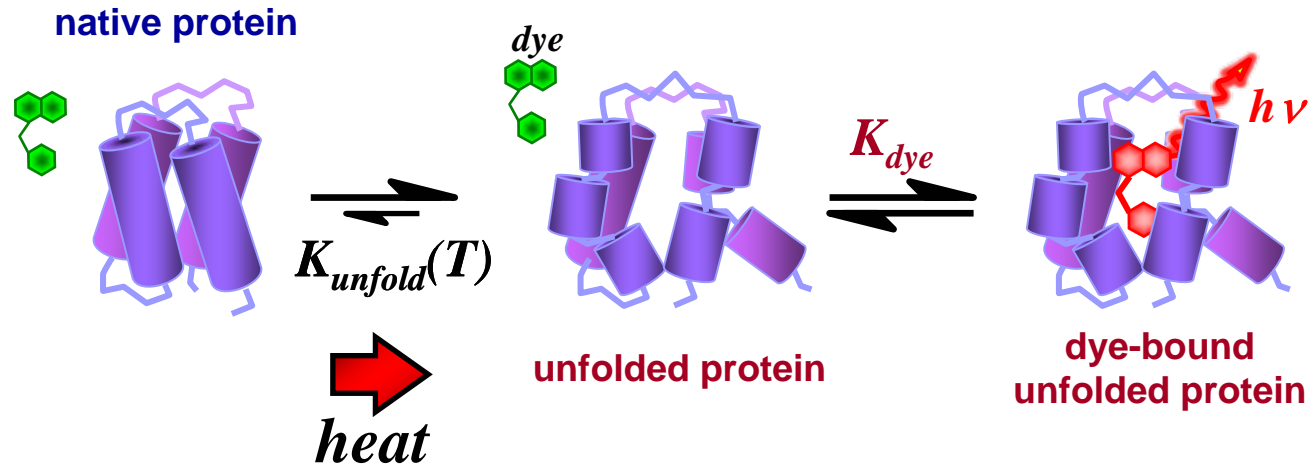


Protein Stability by *ThermoFluor*[®]

Dye-based fluorescence assay of stability



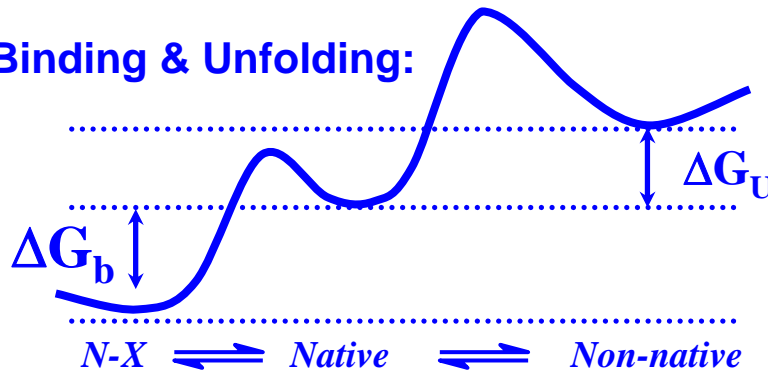
High Temperature drives Unfolding



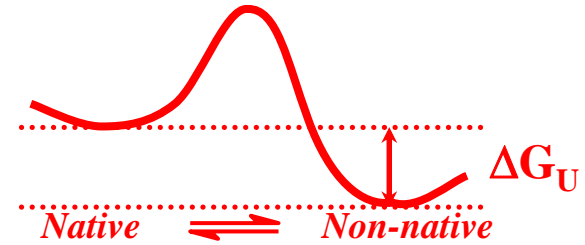
Ligand effect on Thermal Stability

Equilibrium shifts to folded, ligand-bound form

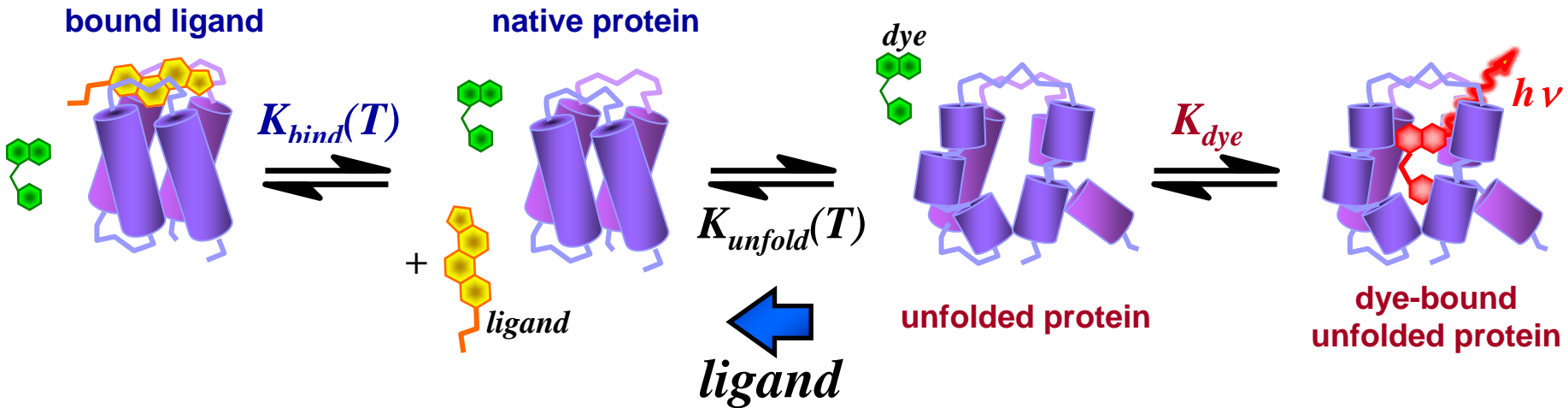
Ligand Binding & Unfolding:



heat



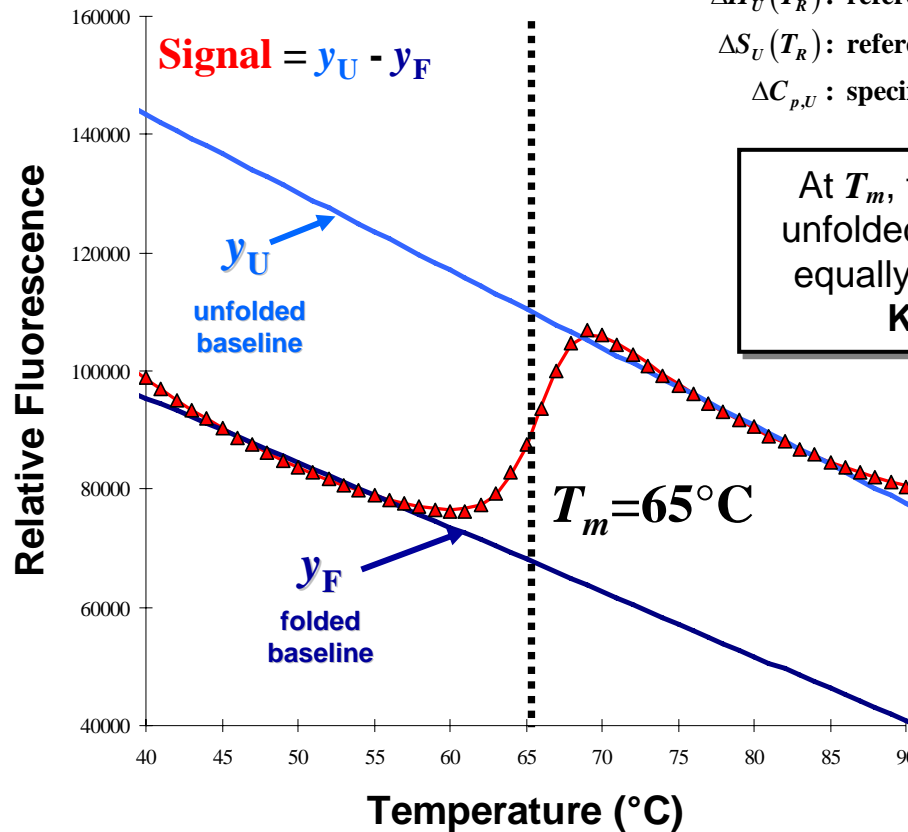
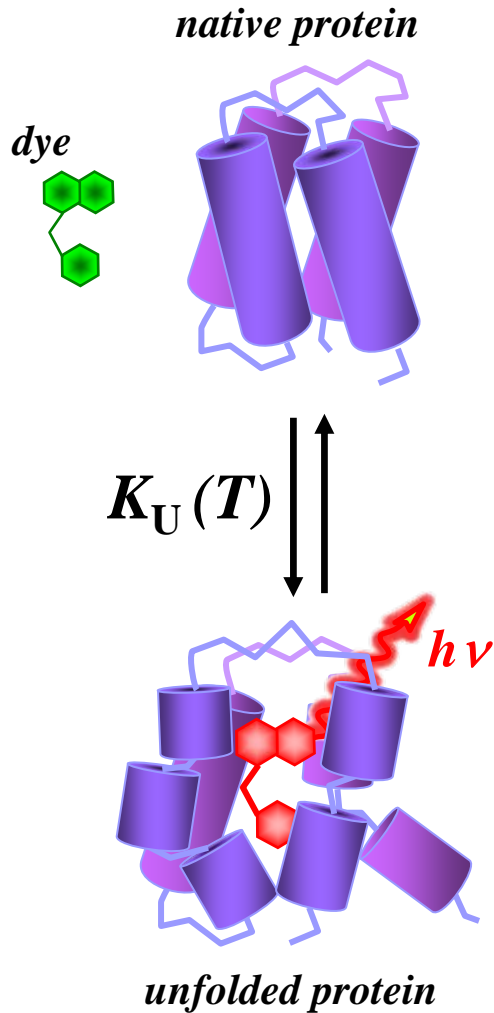
High Temp drives Unfolding



Detailed Fluorescent Melt Parameters

Probability of Unfolding:

$$P_U = \frac{1}{1 + e^{-\{\Delta H_U(T_R) - T\Delta S_U(T_R) + \Delta C_{p,U}[T - T_R - T \ln(T/T_R)]\}/(RT)}}$$



$\Delta H_U(T_R)$: reference enthalpy at $T_R = T_m$

$\Delta S_U(T_R)$: reference entropy at $T_R = T_m$

$\Delta C_{p,U}$: specific heat capacity

At T_m , folded and unfolded states are equally populated

$$K_u = 1$$



ThermoFluor®:

High Throughput Thermodynamic Assay

Plate-based Protein Unfolding

- 384-well assay plate; high throughput characterization and screening of proteins.
- Low volume, 3 μ l, small-scale reactions, ~1 μ M protein; typically < 200 ng well.
- Each well comprises an individual protein unfolding assay.
- Compound binding free energy adds to protein stability – shifts stability curve to higher temperature.

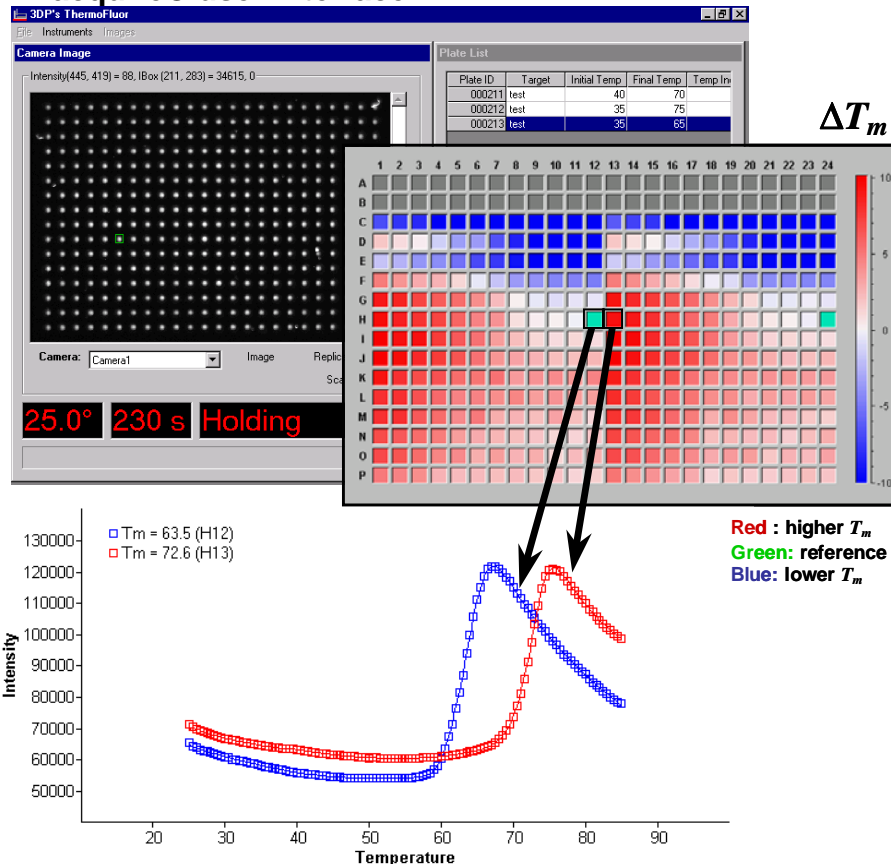
Optimization for HTS is an optimization of protein stability and signal intensity.

M. W. Pantoliano *et al.* (2001) *J. Biomol. Screen.* 6: 429

M. J. Todd & F. R. Salemme (2003) *Gen. Eng. News* 23

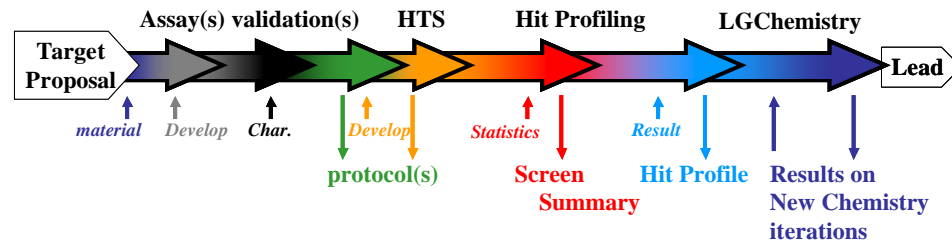
D. Matulis *et al.* (2005) *Biochemistry* 44: 5258

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ThermoFluor[®] in Drug Discovery



❑ Protein Stability Profiling (PSP)

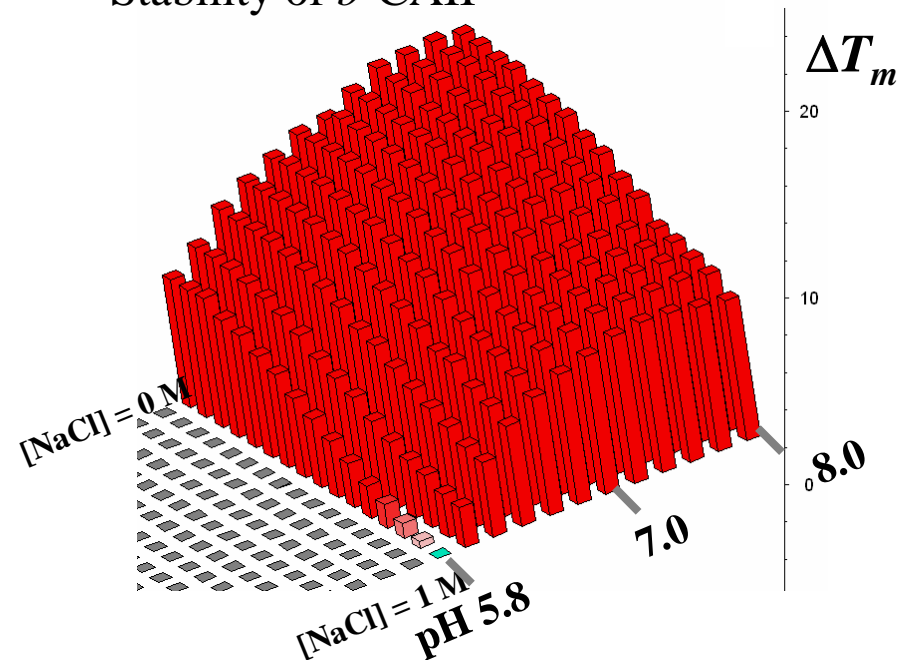
- Protein preparation (pH/Salt, excipient effects)
- Protein crystallography
- Protein Formulation

❑ μ HTS

❑ Hit profiling

- Calculating binding constants
- Triage of “bad” compounds
- Secondary hit profiling
 - Inhibition Mechanisms
 - Competition

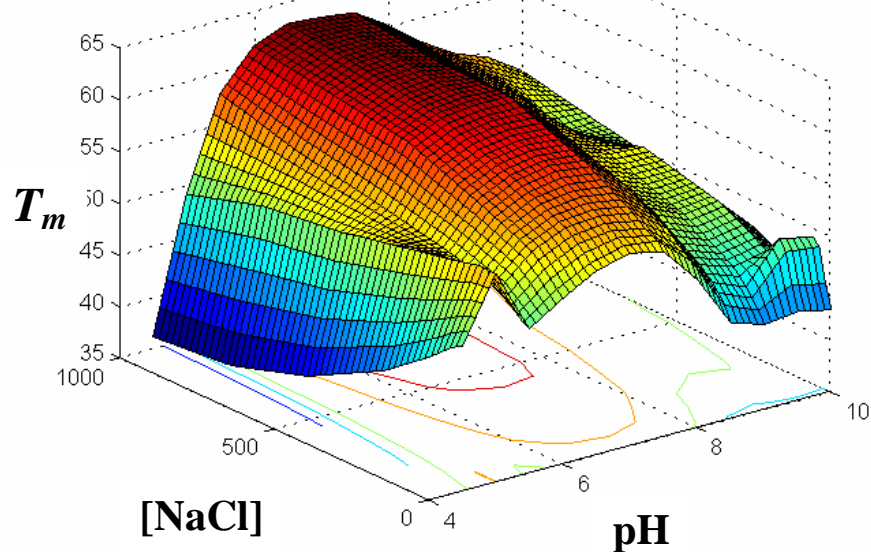
Stability of *b*-CAII



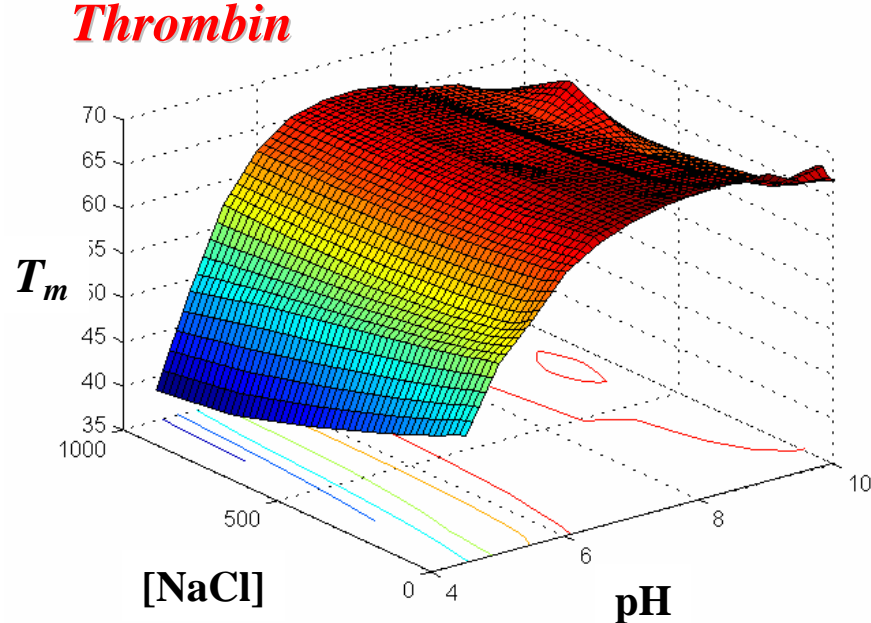
Stability Surfaces of Test Proteins

Variation of T_m with pH and NaCl

Carbonic Anhydrase II



Thrombin



- ❑ Unique stability surface for each protein.
- ❑ Profile is a “fingerprint” for a protein sequence, prep, or formulation.



Array-Based Condition Profiling

pH/Salt Characterization:

- 384-well plate based survey of variable pH & salt conditions.
- Varied in conjunction with arrays of buffer type, $\pm\text{MgCl}_2$.

“Excipient” Characterization:

- Plate-based survey of secondary buffer components:
 - Comparisons of NaCl, KCl, LiCl, NH_4Cl , etc.
 - MgCl_2 vs. MnCl_2 or CaCl_2 ; different anions (Cl^- , SO_4^{-2} , PO_4^{-3})
 - Cosolutes (amines), polyols (glycerol), surfactants (tween20)
 - Essential elements; NiCl_2 , ZnCl_2 , etc.

Ligand Binding & Positive Controls:

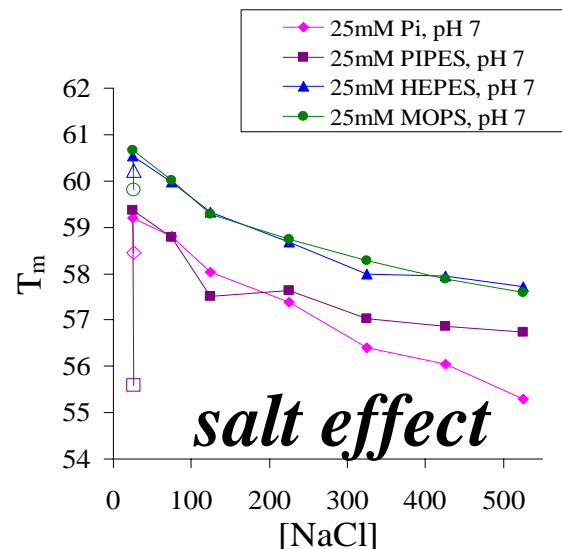
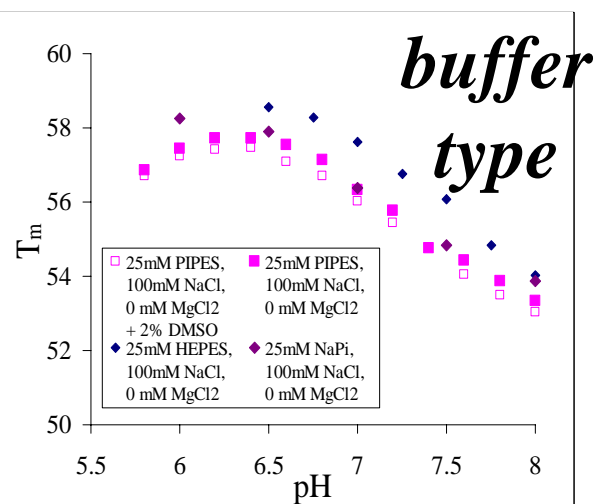
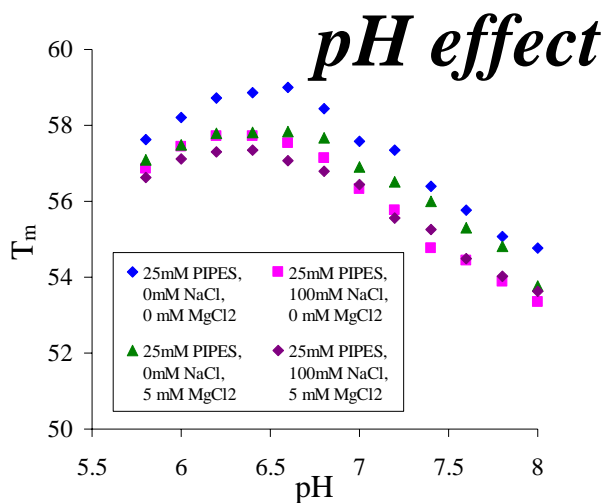
- Direct measurement of ligand binding affinity (dosed compounds).
- Comparison of binding under different conditions (e.g. $\pm\text{MgCl}_2$).

✓ ***Captures Protein-specific Effects in Common Set of SOPs***



Protein Stability Profiling:

Kinase #1 – pH, Salt, & Buffer effects on stability



□ Maximum stability observed ~ pH 6.5

- Screen optimization
- protein preparation implications

□ Mg²⁺ only affects stability at low ionic strength

□ Buffer effects: protein more stable in HEPES than Pi, PIPES

□ Protein stability decreased with high [salt]



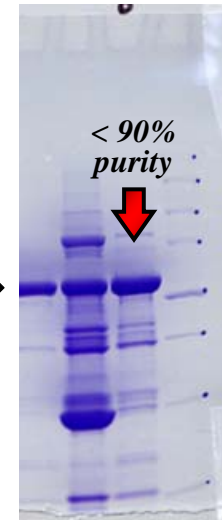
Kinase#1: Protein Purification challenges

□ Expression/Purification

- Expressed as GST-fusion protein
- Purified off GSH-resin, thrombin cleavage
- Described procedure suggests handling at pH 8.0
 - < 90% pure
 - Significant quantities of aggregates present

50mM Tris pH 8.0
150mM NaCl
1mM DTT
10% Glycerol

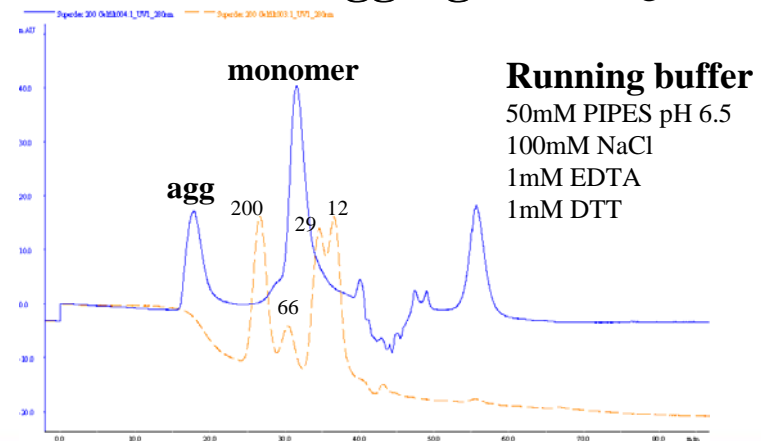
pure protein →



□ PSP suggests

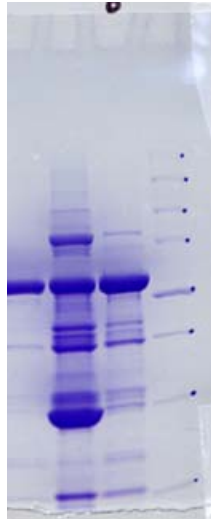
- use lower pH
- use HEPES
- low ionic strength

30% soluble aggregate(1.2mg/ml)



Kinase#1: Protein Purification solutions

pH 8.0
higher salt

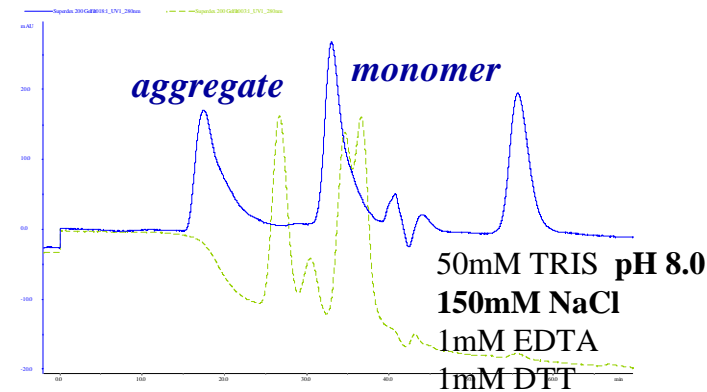


pH 6.8
lower salt
10% glycerol

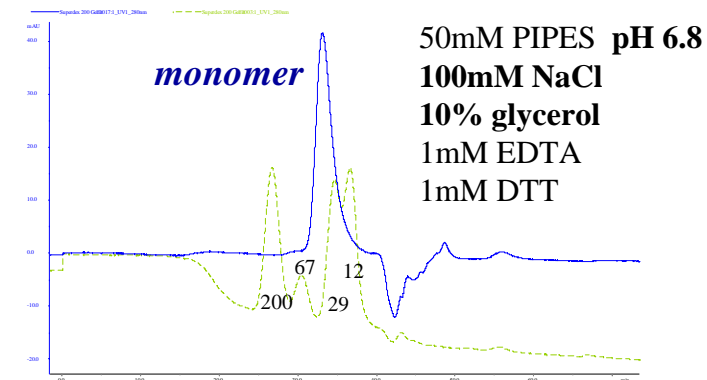


protein band →

Original (Published) Protocol



Revised Procedure



□ Using conditions from PSP

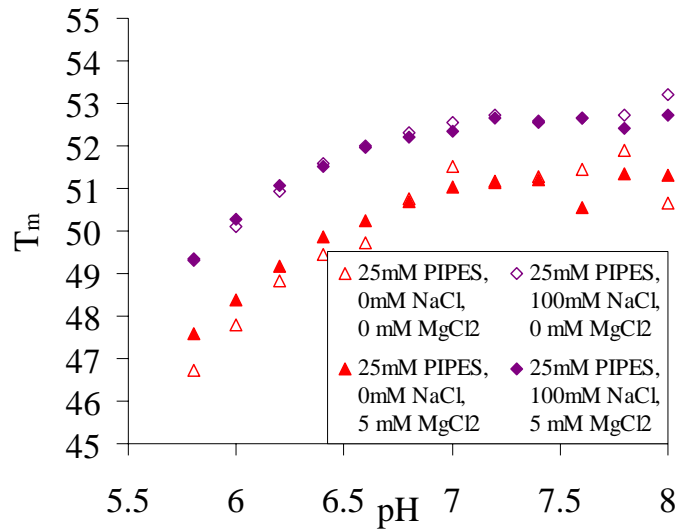
- altered thrombin cleavage kinetics
- significantly improved protein purity
- prevented aggregate formation



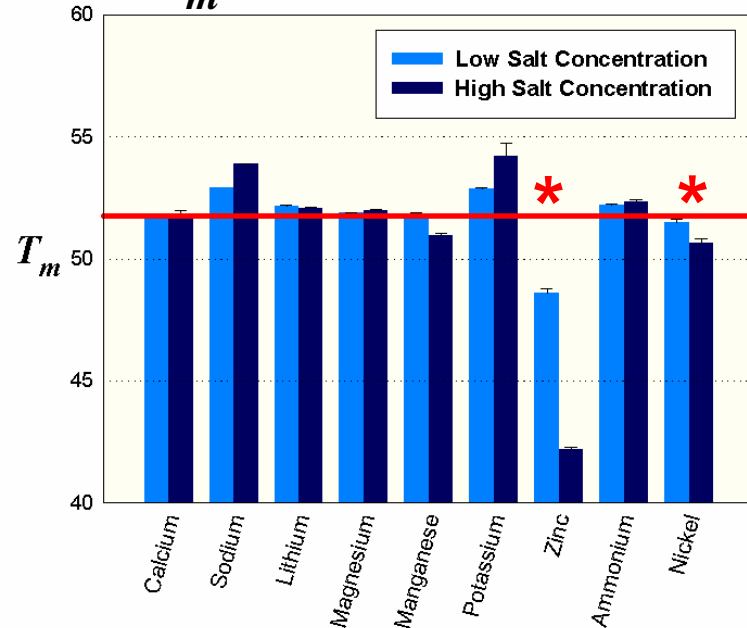
Protein Stability Profiling:

Kinase #2 – pH, Salt, & Buffer effects on stability

pH ± NaCl & MgCl₂



T_m vs. Chloride Salts

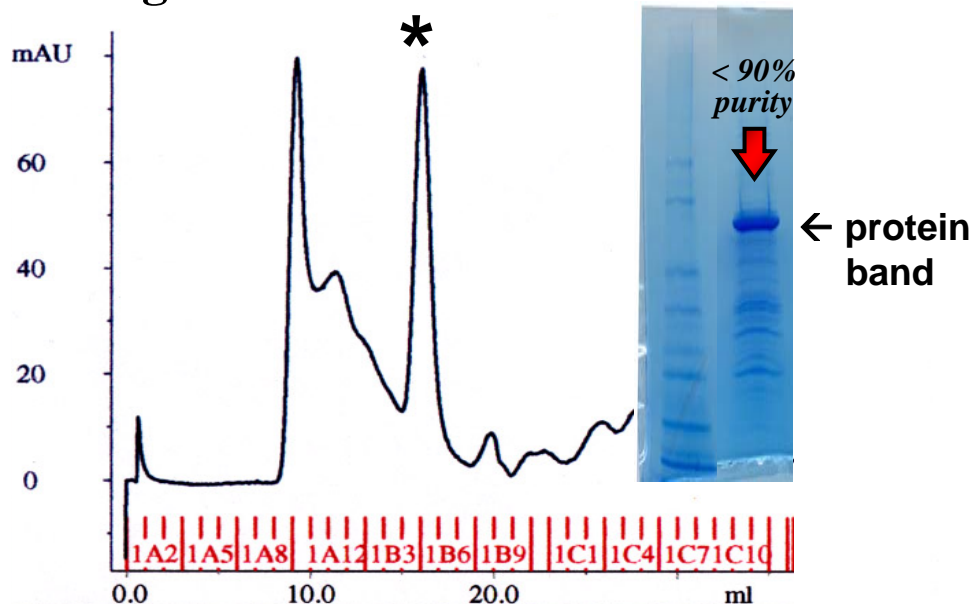


- High Salt stabilizes the kinase domain (also Phosphate Buffer).
- Protein is destabilized by Zinc and by Nickel (also imidazole).
- Combination of NiCl₂ & HEPES Buffer used initially in prep.**



Kinase #2: Protein Purification Challenges

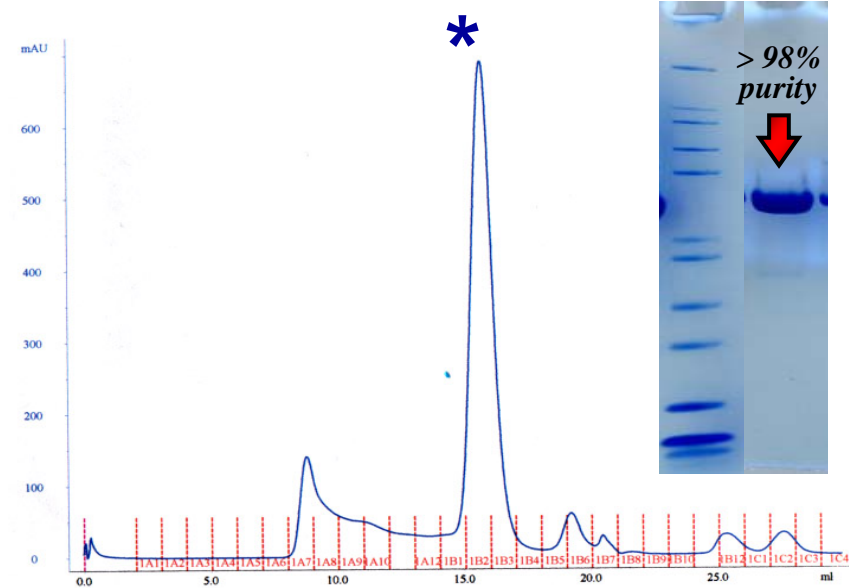
Original Protocol



Gel filtration analysis of protein eluted from Nickel-NTA column in HEPES buffer.

* peak corresponding to gel fraction.

Revised Procedure



Gel filtration analysis of protein eluted from Talon column in phosphate buffer.

* peak corresponding to gel fraction.

Change of column type minimized exposure of protein to Nickel.



Kinase Protein Stability Profiling

Kinase #1

- ❑ Original conditions:
 - Tris Buffer, typical salt & reductant, GST-column purification
 - Aggregation was biggest challenge

- ❑ Protein Stability Profile:
 - pH profile - maximum at pH ~ 6.5
 - Salt profile – prefers low salt, polyols
 - Buffer profile – HEPES preferable to Phosphate, PIPES, MOPS
 - Metals - divalents are destabilizing

- ❑ PSP-Altered Purification:
 - Changed to HEPES Buffer
 - Added 10% Glycerol to thrombin cleavage & column elution buffer
 - Minimized Aggregation

Kinase #2

- ❑ Original conditions:
 - HEPES Buffer, typical salt, Nickel-column purification
 - Aggregation was biggest challenge

- ❑ Protein Stability Profile:
 - pH profile - maximum at pH > 7
 - Salt profile – stabilized by high salt
 - Buffer profile - Phosphate buffers uniquely stabilizing
 - Metals - Nickel is destabilizing

- ❑ PSP-Altered Purification:
 - Changed to Phosphate Buffer
 - Substituted Talon Column for Ni-NTA column
 - Minimized Aggregation



Enzyme Assay Development

Target Characterization at a Basic Level

Well-studied System

- Establish correct form of enzyme/substrates.
- Signal Optimization.
- Effects of buffer (pH, salt, etc) and temperature on activity.
- Measure K_m 's, K_d 's, EC_{50} 's for all substrates & cofactors.
- Measure *true* V_{max} ; k_{cat} where feasible.
- Measure K_i 's/ IC_{50} 's for known inhibitors.

Poorly-characterized System (additional work)

- Investigate a minimum set of potential biological substrates.
- Test all known assays.
- Screen additives/ligands to investigate affects on activity.
- Detailed kinetic characterization (establish kinetic mechanism).
- Mechanistic studies for inhibitors and tool compounds (determine true K_i).



Enzyme Assay Development: Streamlined Characterization Approach

Signal Optimization

- 1) Wavelength(s)
- 2) Rate/Enz. Conc.

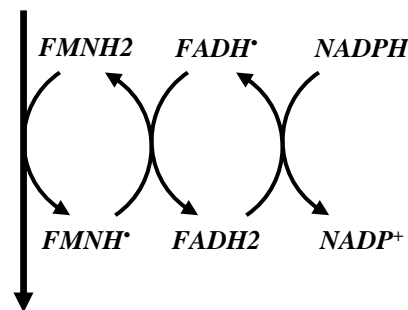
Condition Profiling

- 1) pH & Salt
- 2) excipients

Optimization for Automation

Challenging System:

Substrates

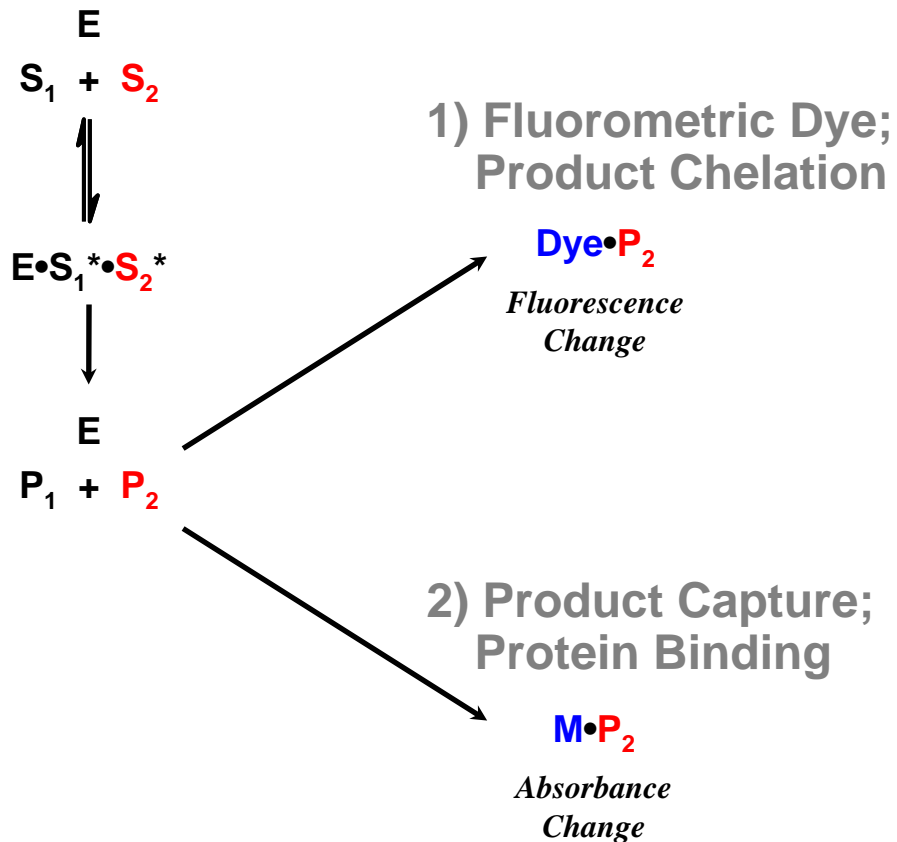


Products

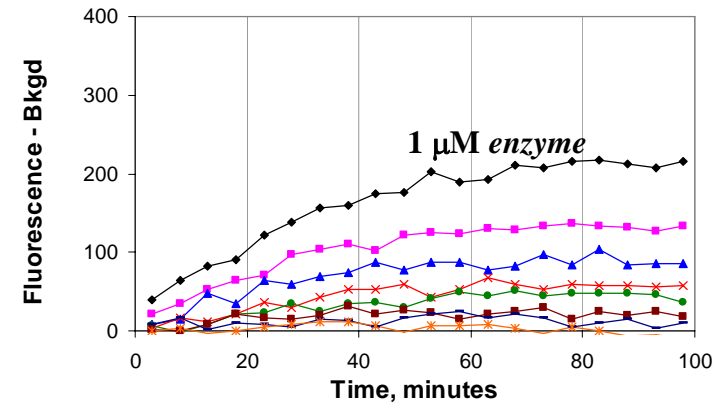
- Reductase/Oxidase Activities; multi-step enzyme mechanism.
- One of the Products is Transiently Stable – opportunity for capture.



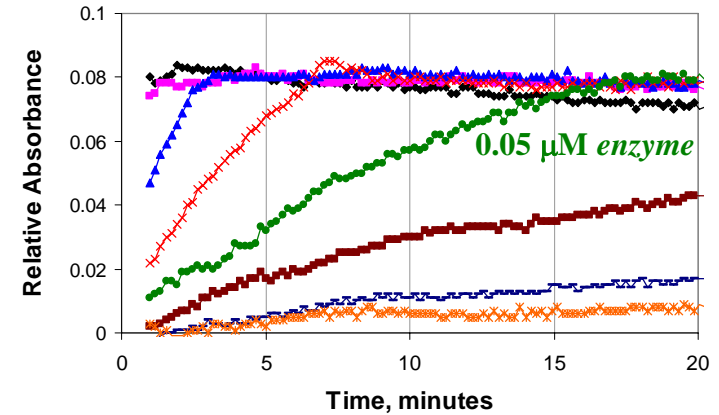
Enzyme Assay Development: Rate-based Product Detection Assays



Dye Chelation Assay:
Variable [Enzyme] - 5 to 1000 nM

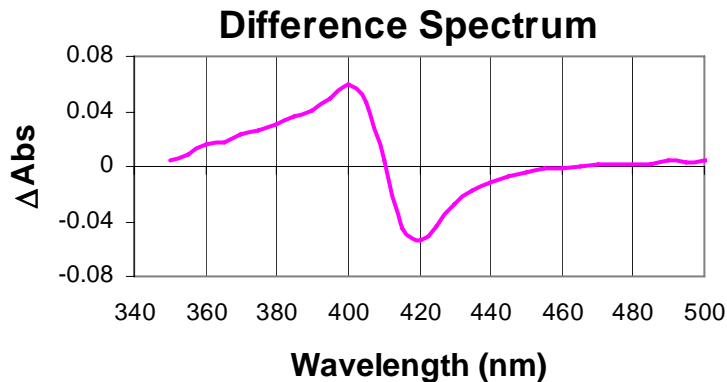
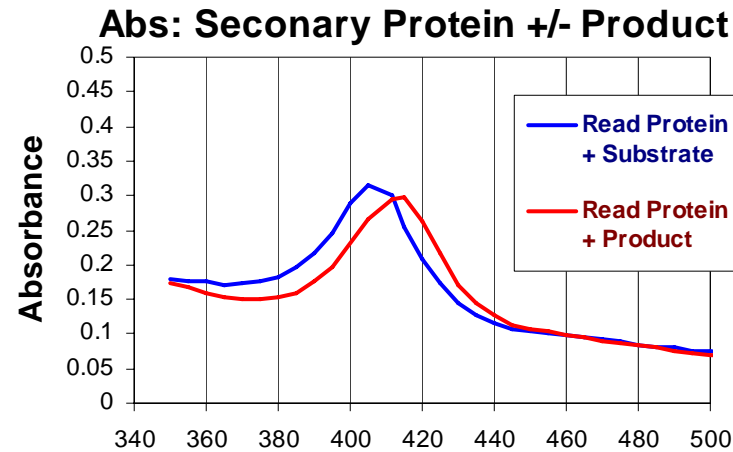


Secondary Binding Assay:
Variable [Enzyme] - 5 to 1000 nM

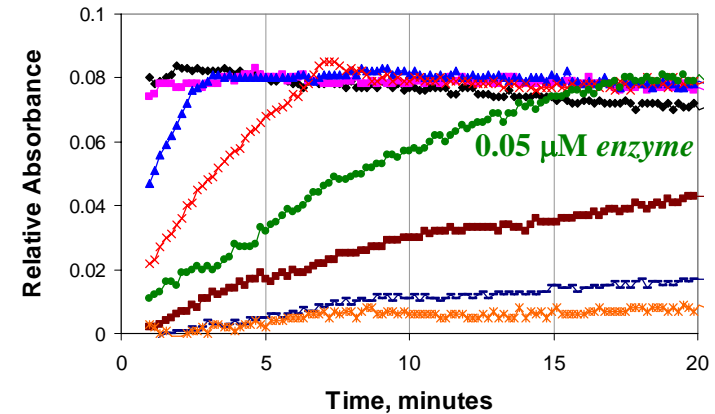


Signal Optimization

Dual Wavelength Absorbance Assay



**Secondary Binding Assay:
Variable [Enzyme] - 5 to 1000 nM**



- Wavelength-difference (400 – 420nm):
 - Double Signal of single wavelength.
 - Additional Signal Stability.



Conditional Effects on Rx Rates

Array-based Approach:

Survey of pH & Salt:

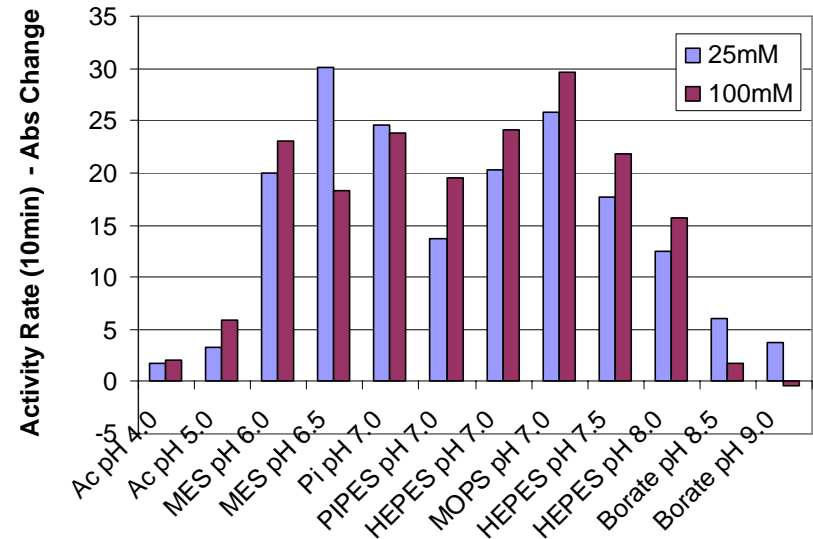
- The activity rates (after 10min) in the are similar from pH 6.0-7.5.
- Initial rates are similar between 100-400 mM NaCl

Excipient effects on Rates:

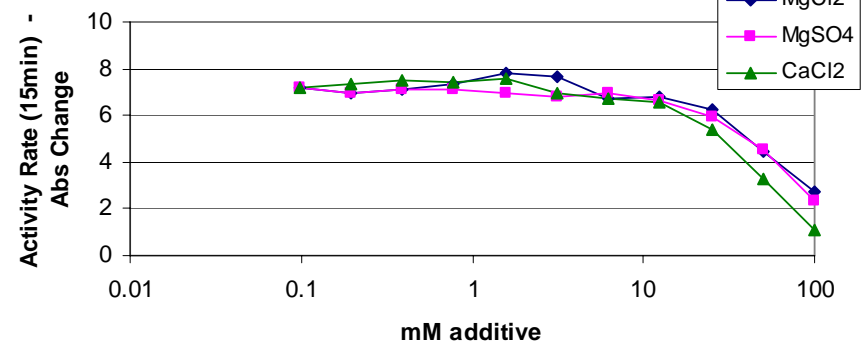
- Increased Activity Rates: CaCl_2 , MgCl_2 , Tween 20
- Significantly Decreased Rates: NiSO_4 , PEG, Imidazole
- Tween20 optimal at 0.01%; DMSO tolerated up to 2%

❑ Buffer modified to HEPES, pH7; CaCl_2 , MgSO_4 , Tween, GSH added to minimize [Enzyme].

Buffer Plate - 100nM Enzyme



Titration of Excipients



Initial Automation Uniformity Tests

Zprime vs [Enzyme] – 10 min. endpoint read (384):

[Enzyme]	Signal Mean	Signal Std	BG Mean	BG Std	Signal:BG	Zprime
60nM	12.59	0.51	0.43	0.13	30	0.90
40nM	9.21	0.44	0.36	0.15	25	0.90
30nM	6.39	0.45	0.39	0.20	16	0.88
*20nM	4.38	0.27	0.26	0.12	17	0.89
10nM	2.26	0.25	0.22	0.15	10	0.84
<i>5nM</i>	<i>1.35</i>	<i>0.35</i>	<i>0.17</i>	<i>0.11</i>	<i>8</i>	<i>0.38</i>

**Screening Concentration – going forward in 1536 for uHTS*

- Uniform Z' > 20 nM enzyme, with slight decrease at 10 nM (first pass).
 - Signal becomes limiting at the lowest enzyme concentration.
 - Stability of endpoint read is high in longevity tests
(Z' > 0.8 after 2 hours on ice and > 0.65 after 4 hours at room temperature)
- ✓ ***Once conditions optimized from Standardized Profiling, no additional optimization needed for screening.***



Summary

Protein Stability and Functional Profiling

- General, homogeneous assays are powerful tools to assay protein stability and function.
 - Easy to tune conditions to a single protein vs. a survey of protein constructs (truncations/mutations).
 - Routine improvement in yields, purity, and minimized aggregation in recombinant protein preps.

- Similar, broad assay characterization can be readily applied to functional/enzyme assays.
 - Systemized set of questions/processes related to source of signal, variations in activity, and system variables.
 - “Growing pains” associated with transfer to robotics are minimized when protein mechanism is well characterized.



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Marina Nelen

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