Streamlining Assay Development:

Lessons in Process Optimization Through Protein Optimization

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Lead Generation Biology

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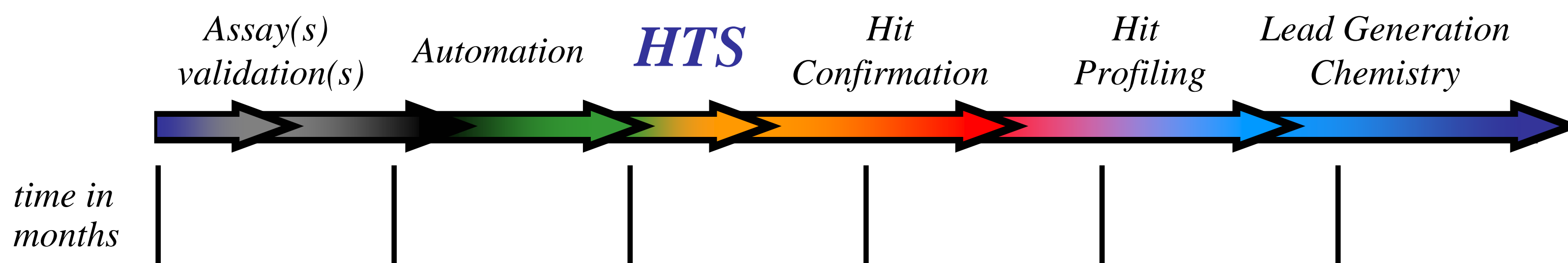
Rate Limiting Steps in Lead Generation

- ❑ 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.

Cellular & *in vivo*, assays
second messenger effects
upregulation/downregulation
gene activation/repression
ADME/Toxicity

Molecular "activity" assays
inhibition (IC50)
competitive binding
ELISAs
signal transduction pathways
enzyme mechanisms

Biophysical assays
structural (X-ray, NMR)
binding
thermodynamic
in silico (predictive) methods
spectroscopic (CD, Fluor., scattering)

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.

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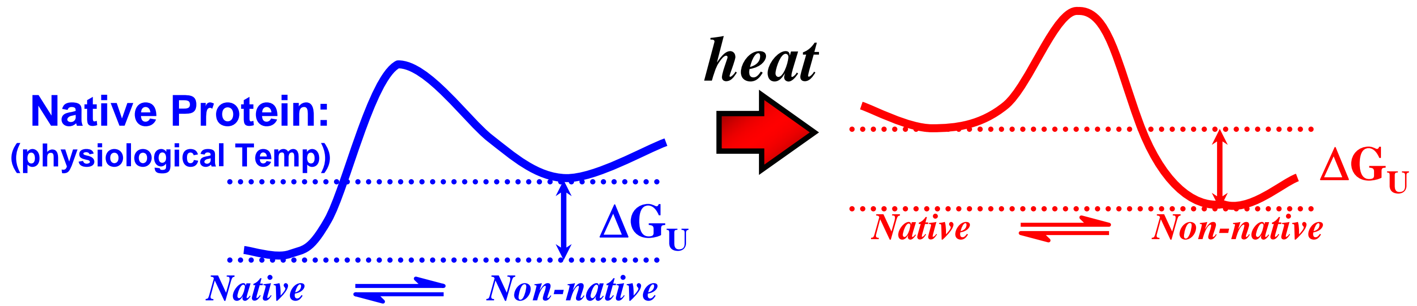
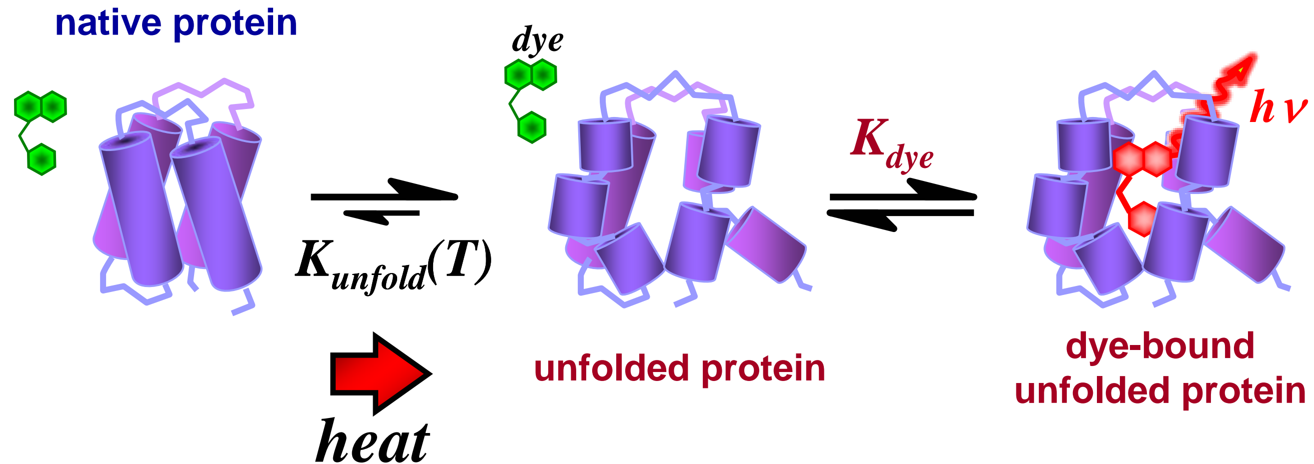
Protein Stability by *ThermoFluor®*

Dye-based fluorescence assay of stability

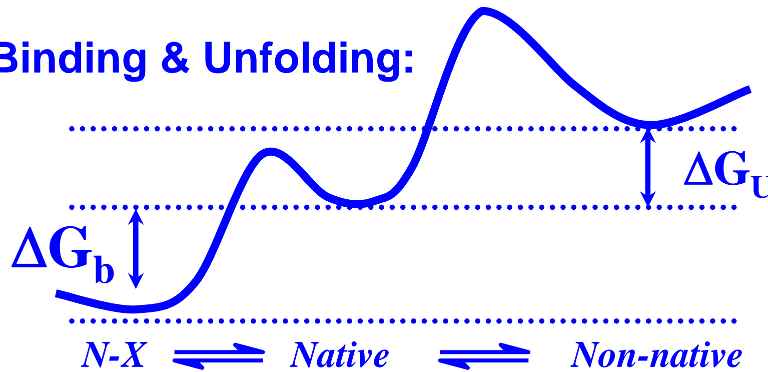


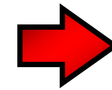
High Temperature drives Unfolding

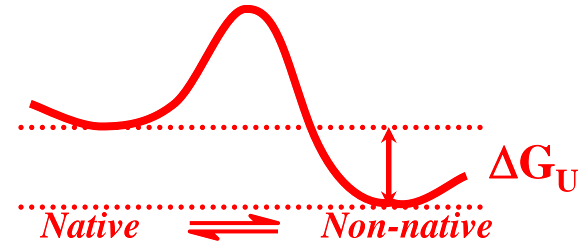


Ligand effect on Thermal Stability

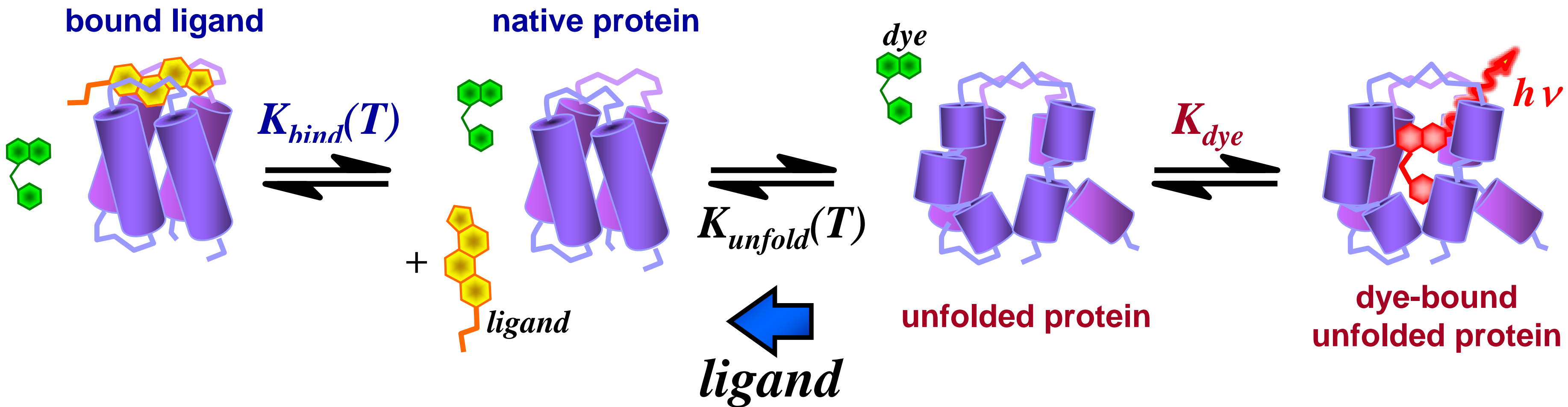
Equilibrium shifts to folded, ligand-bound form



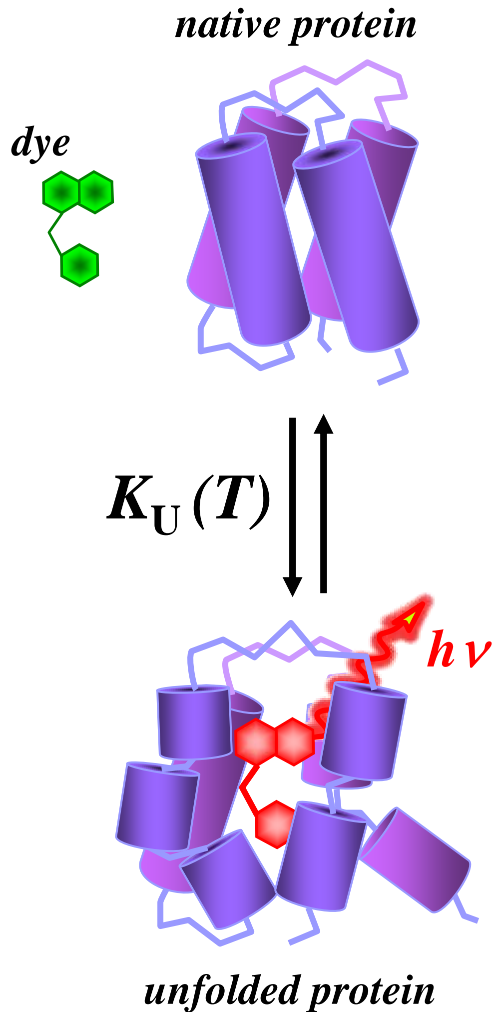




High Temp drives Unfolding



Detailed Fluorescent Melt Parameters



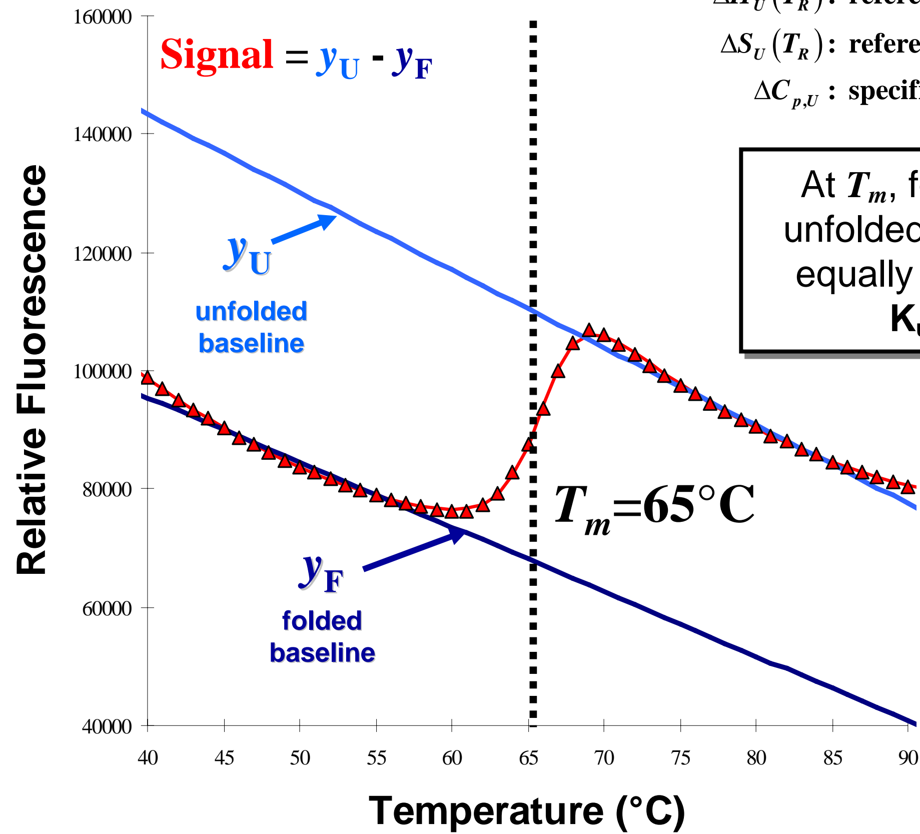
Probability of Unfolding:

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

$\Delta H_U(T_R)$: 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

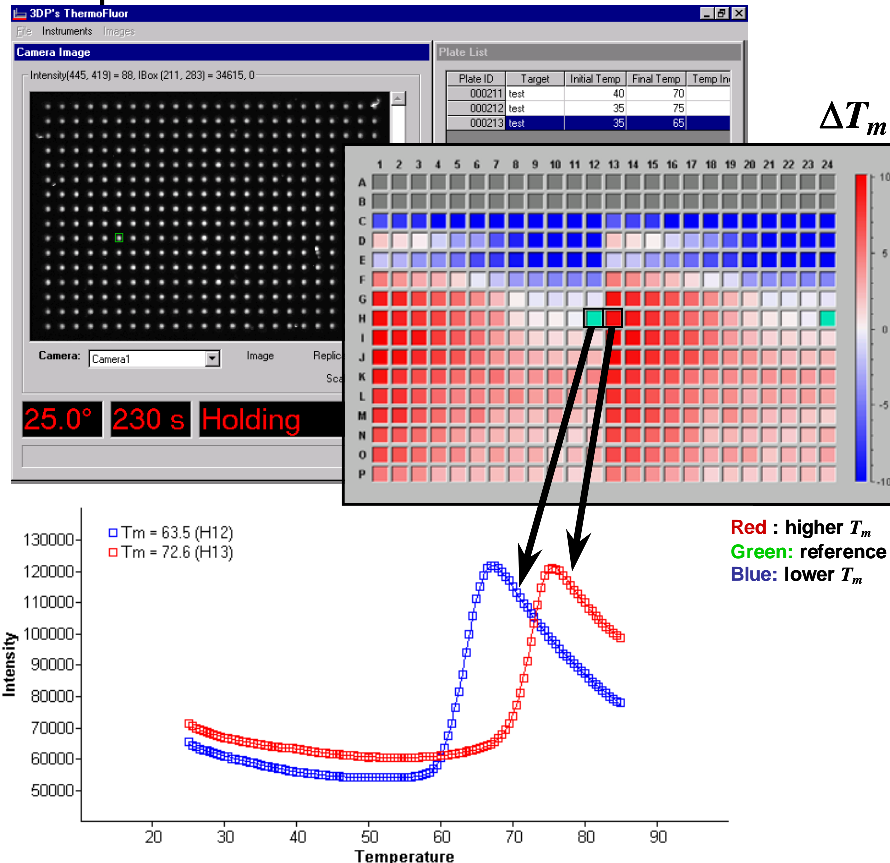
ThermoFluor®: High Throughput Thermodynamic Assay

Plate-based Protein Unfolding

- 384-well assay plate; high throughput characterization and screening of proteins.
- Low volume, 3 µl, small-scale reactions, ~1 µM 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.

TFacquire® user interface



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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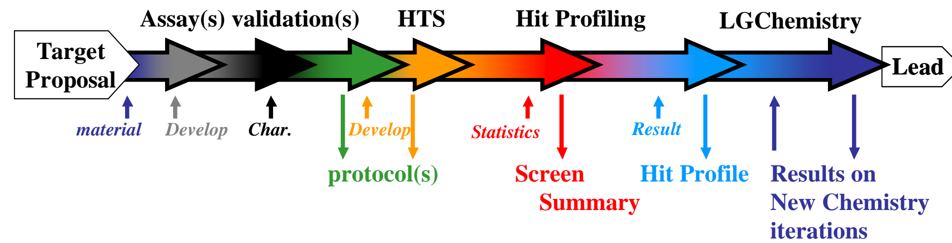
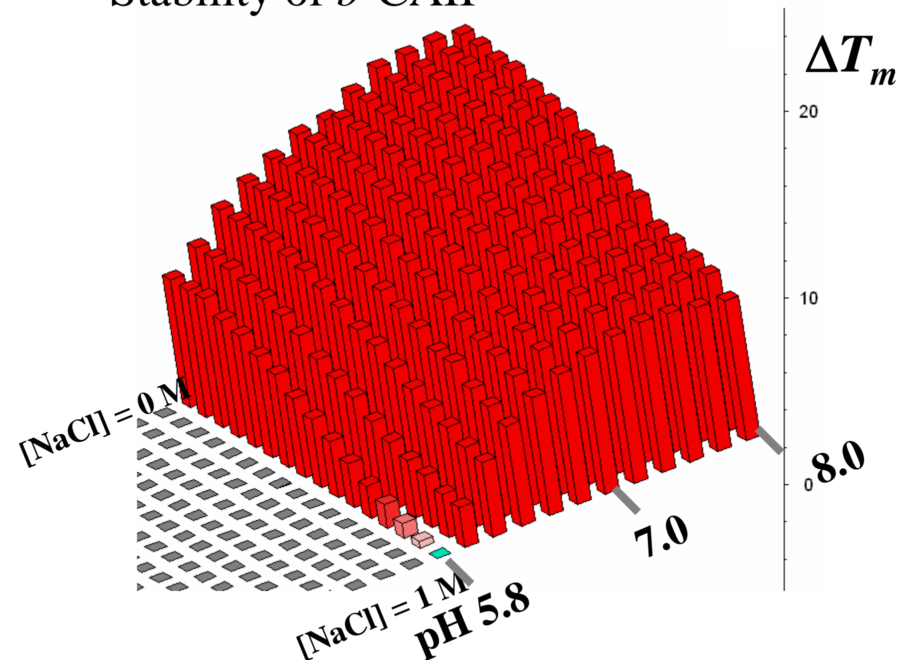
ThermoFluor® in Drug Discovery

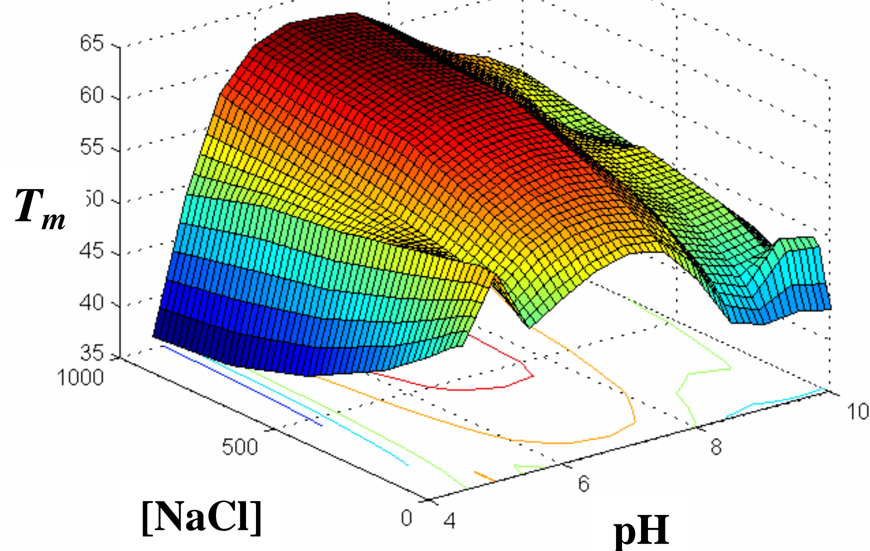


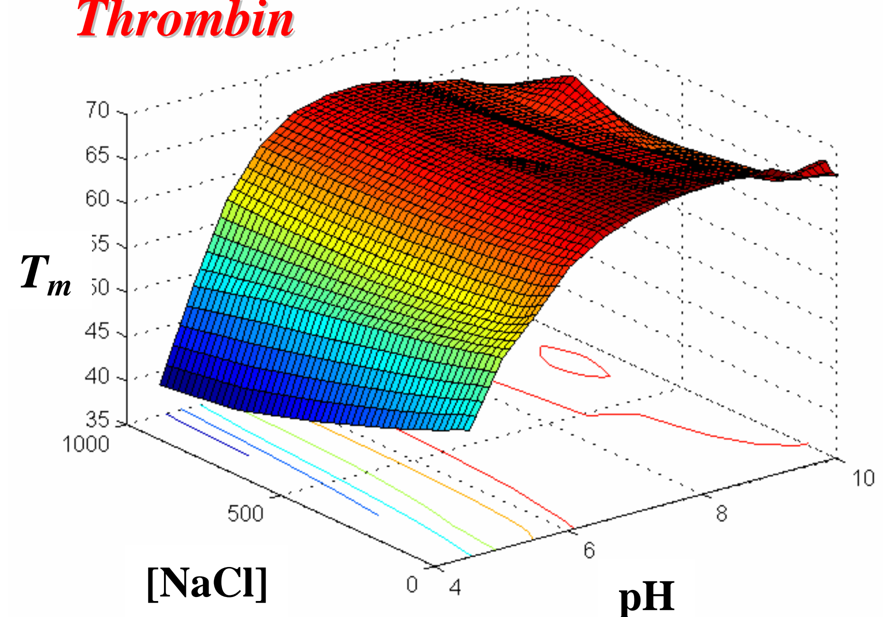
- Protein Stability Profiling (PSP)
  - Protein preparation
    (pH/Salt, excipient effects)
  - Protein crystallography
  - Protein Formulation
- μHTS
- Hit profiling
  - Calculating binding constants
  - Triage of "bad" compounds
  - Secondary hit profiling
    - Inhibition Mechanisms
    - Competition



Stability Surfaces of Test Proteins

Variation of T_m with pH and NaCl





- 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, ±$MgCl_2$.

"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. ±$MgCl_2$).

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

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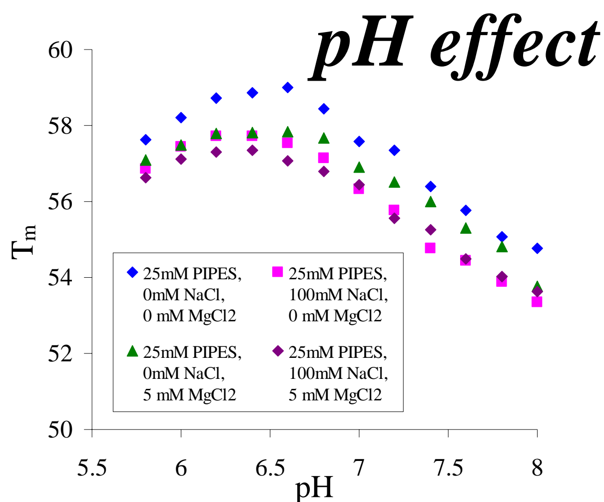
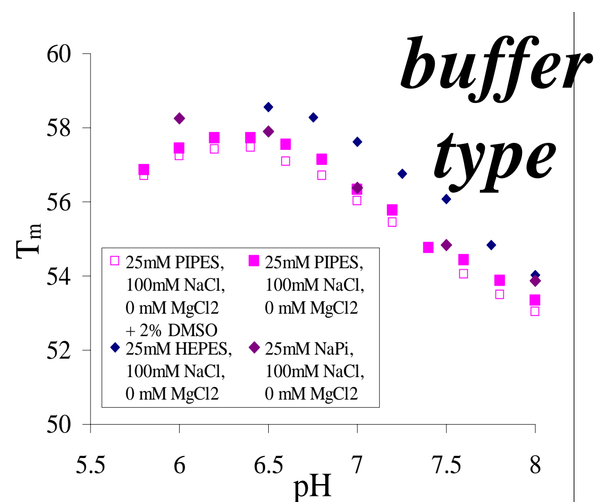
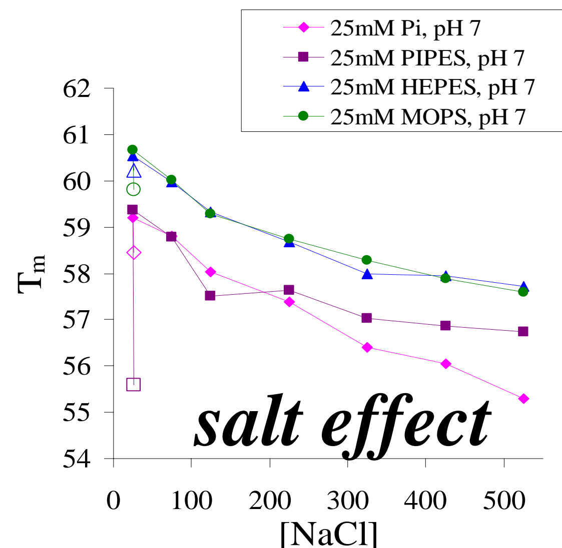
Protein Stability Profiling:

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







- **Maximum stability observed ~ pH 6.5**
  - Screen optimization
  - protein preparation implications
- **Mg^{2+} only affects stability at low ionic strength**
- **Buffer effects: protein more stable in HEPES than Pi, PIPES**
- **Protein stability decreased with high [salt]**

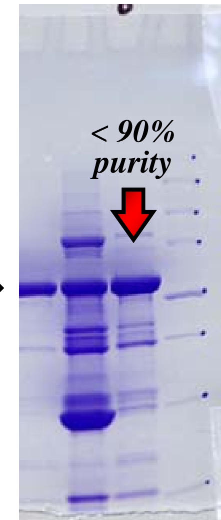
Kinase#1: Protein Purification challenges

- 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

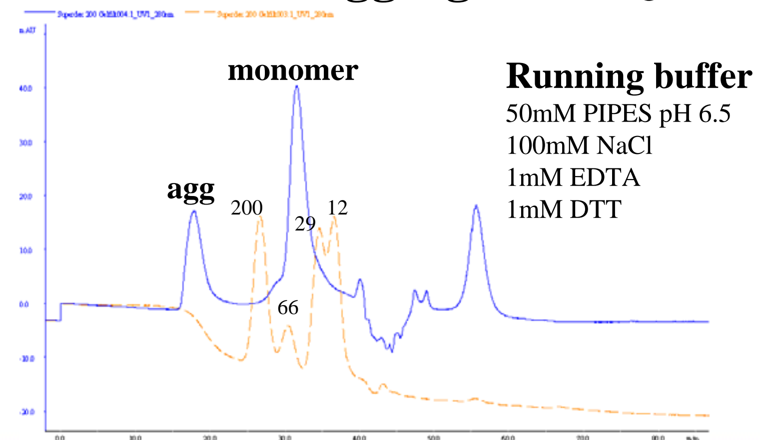
- PSP suggests
  - use lower pH
  - use HEPES
  - low ionic strength

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

pure protein →



30% soluble aggregate(1.2mg/ml)

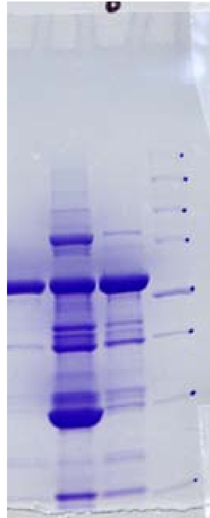


Kinase#1: Protein Purification solutions

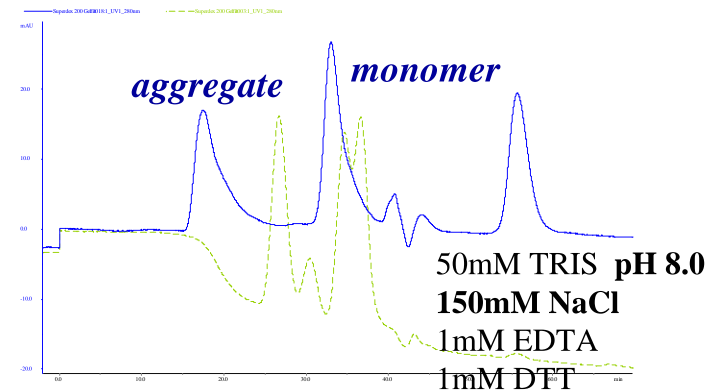
pH 8.0
higher salt

pH 6.8
lower salt
10% glycerol

protein band →

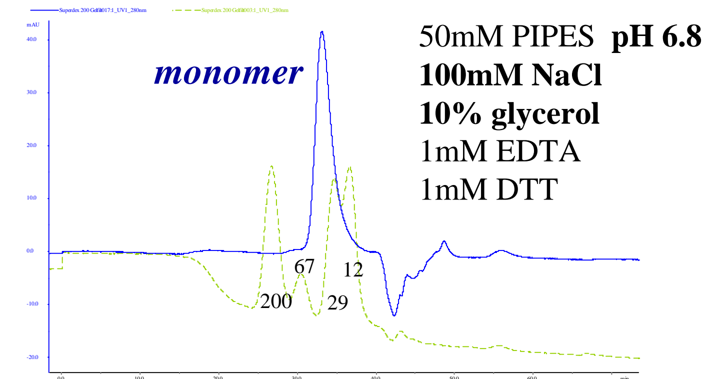


Original (Published) Protocol



50mM TRIS **pH 8.0**
150mM NaCl
1mM EDTA
1mM DTT

Revised Procedure



50mM PIPES **pH 6.8**
100mM NaCl
10% glycerol
1mM EDTA
1mM DTT

- Using conditions from PSP
  - altered thrombin cleavage kinetics
  - significantly improved protein purity
  - prevented aggregate formation

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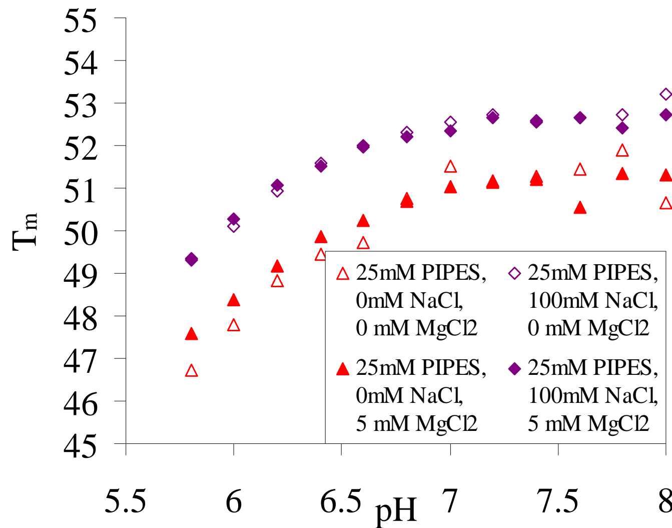
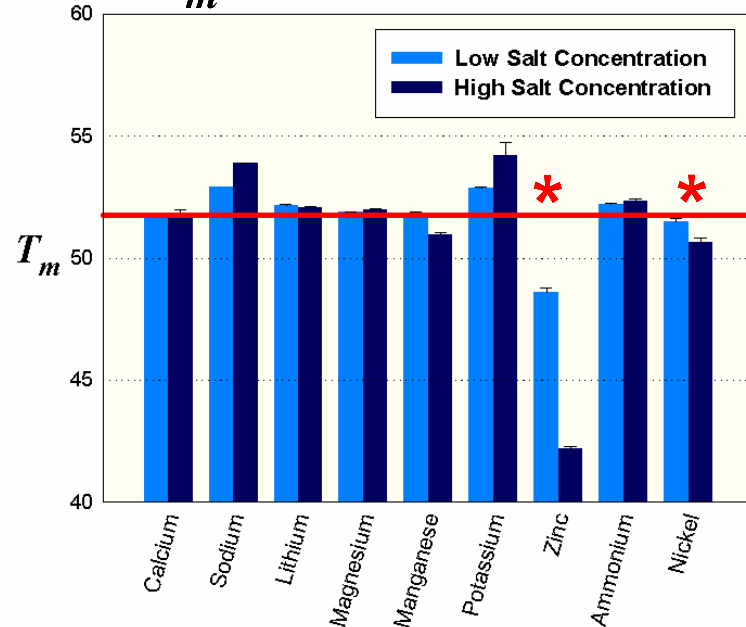
Protein Stability Profiling:

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

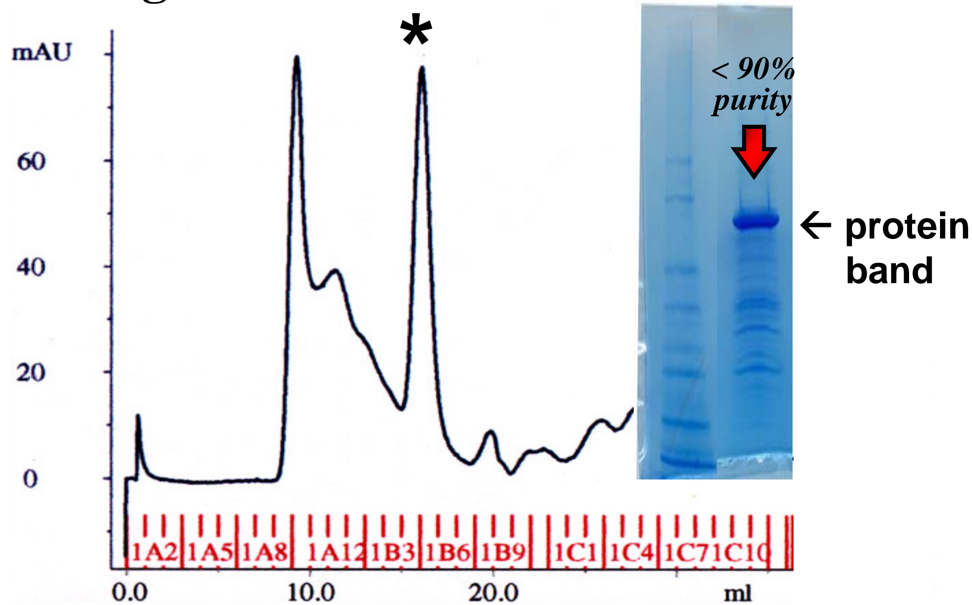




- High Salt stabilizes the kinase domain (also Phosphate Buffer).
- Protein is destabilized by Zinc and by Nickel (also imidazole).
- Combination of NiCl2 & HEPES Buffer used initially in prep.

Kinase #2: Protein Purification Challenges

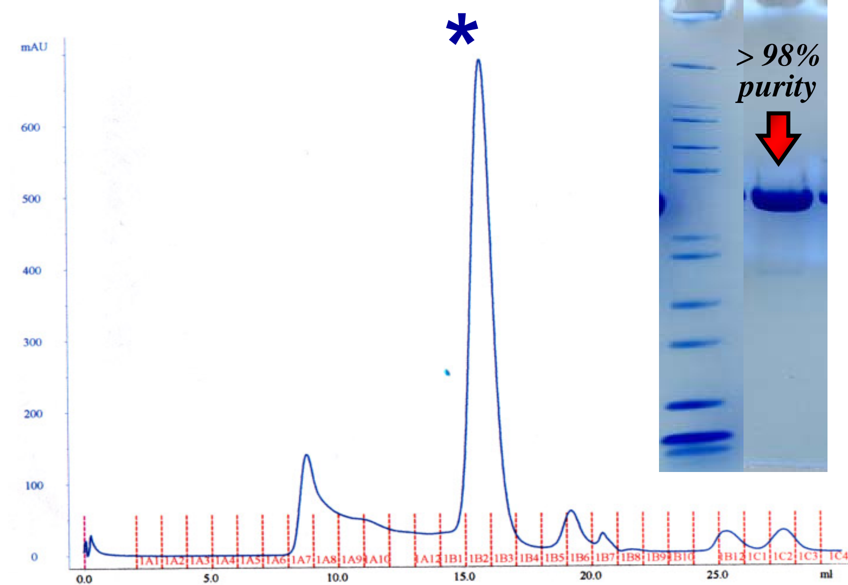
Original Protocol



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.**

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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 Km's, Kd's, EC50's for all substrates & cofactors.
- Measure *true* Vmax; kcat where feasible.
- Measure Ki's/IC50'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 Ki).

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Enzyme Assay Development:
Streamlined Characterization Approach

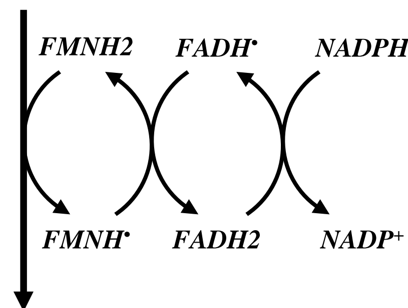
Signal Optimization
1) Wavelength(s)
2) Rate/Enz. Conc.

Condition Profiling
1) pH & Salt
2) excipients

Optimization for Automation

Challenging System:

Substrates

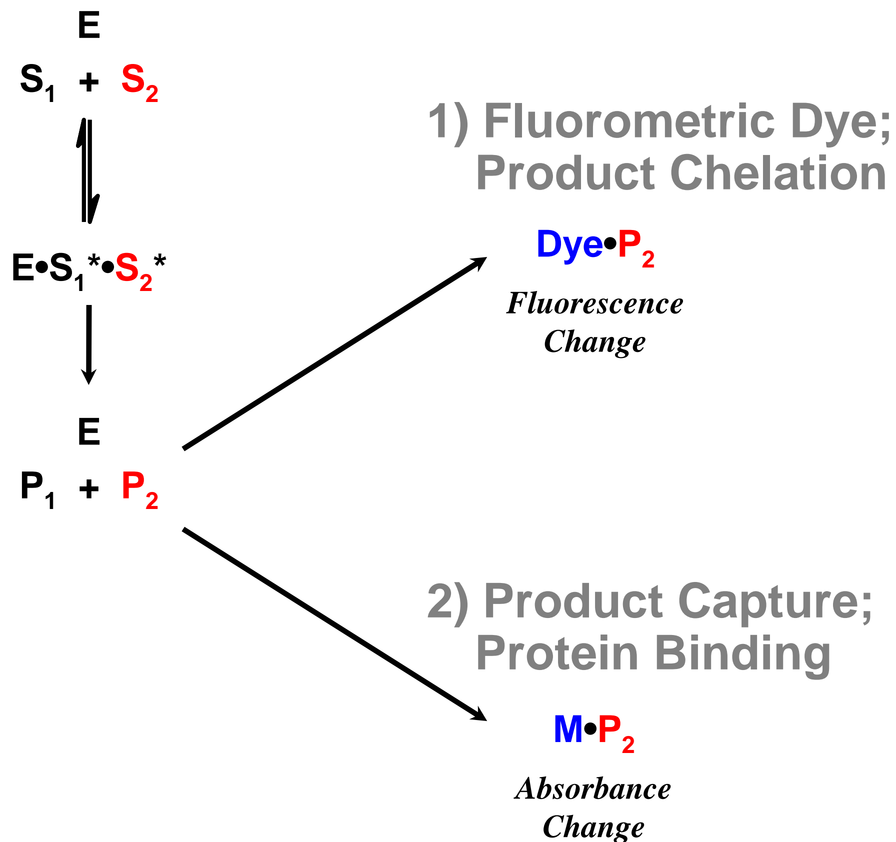


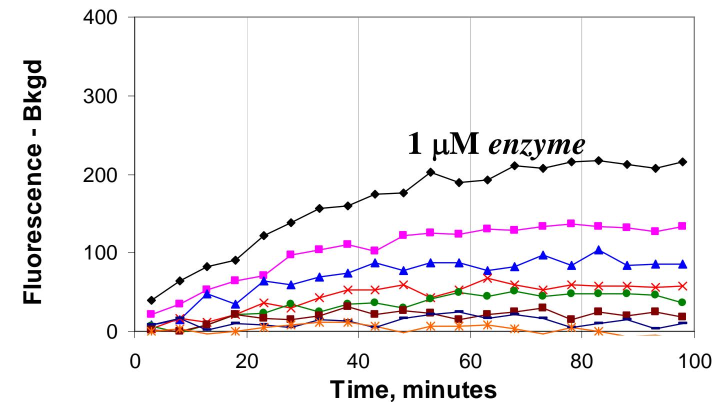
Products

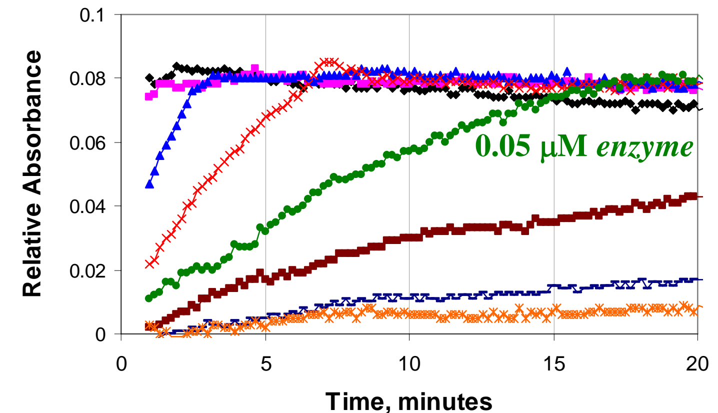
- Reductase/Oxidase Activities; multi-step enzyme mechanism.
- One of the Products is Transiently Stable – opportunity for capture.

Enzyme Assay Development:

Rate-based Product Detection Assays

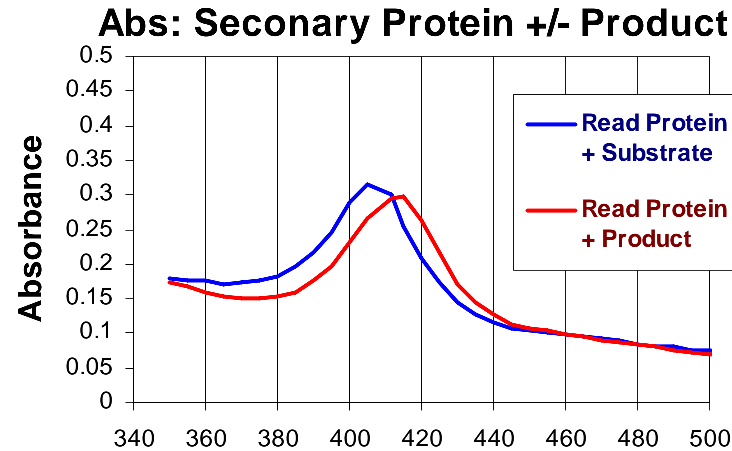


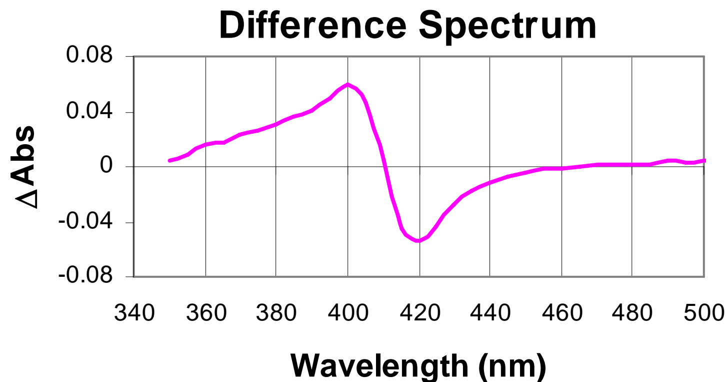


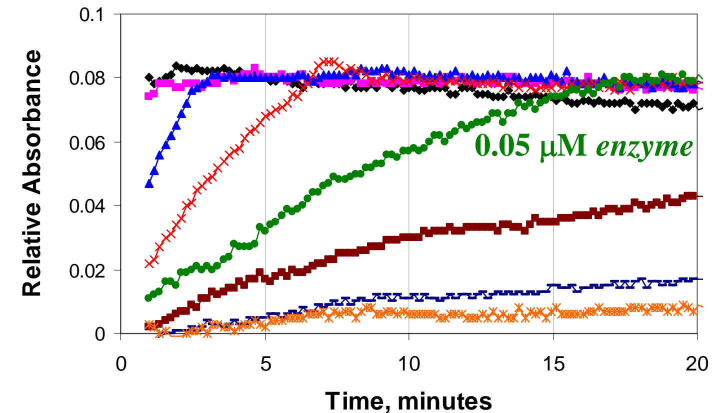


Signal Optimization

Dual Wavelength Absorbance Assay







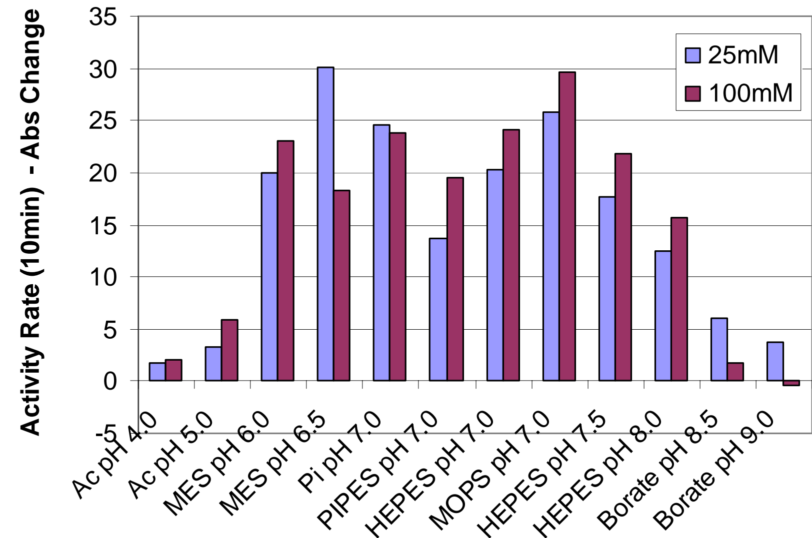
- Wavelength-difference (400 – 420nm):
  - Double Signal of single wavelength.
  - Additional Signal Stability.

Conditional Effects on Rx Rates

Array-based Approach:

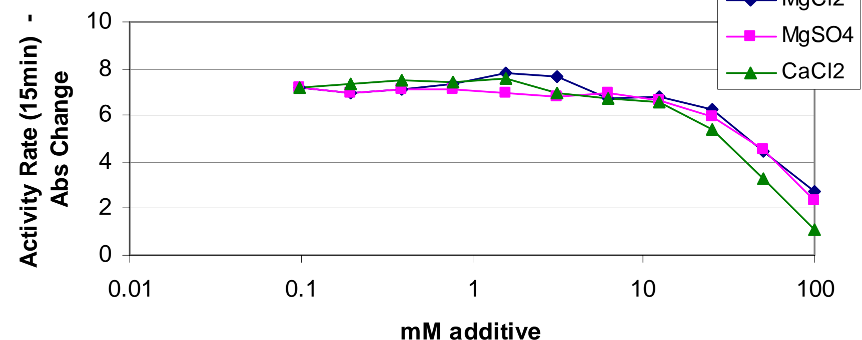
Survey of pH & Salt:

- 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: NiSO4, 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].

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
5nM	*1.35*	*0.35*	*0.17*	*0.11*	*8*	*0.38*

**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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