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 $\Delta G$ (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

Native Protein: (physiological Temp)

Native $\rightarrow$ Non-native

Δ$G_U$

Native $\rightleftharpoons$ Non-native

Δ$G_U$

Native Protein:

Native protein

$dye$

$dye$-bound unfolded protein

$K_{unfold}(T)$

$K_{dye}$

$heat$

unfolded protein

dye-bound unfolded protein

$heat$
Ligand effect on Thermal Stability

*Equilibrium shifts to folded, ligand-bound form*

- **Ligand Binding & Unfolding:**
  - $\Delta G_b$
  - $N-X \rightleftharpoons \text{Native} \rightleftharpoons \text{Non-native}$

- **High Temp drives Unfolding**
  - $\Delta G_U$

- **Heat**

- **$K_{bind}(T)$**
  - Bound ligand
  - Native protein

- **$K_{unfold}(T)$**
  - + ligand

- **$K_{dye}$**
  - dye
  - Unfolded protein

- **Ligand Binding & Unfolding:**
  - dye-bound unfolded protein
  - dye-bound native protein
Detailed Fluorescent Melt Parameters

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

- \( \Delta H_U(T_R) \): 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 \( K_U = 1 \)

\[ \text{Signal} = y_U - y_F \]

\( K_U(T) \)

\( h \nu \)

Relative Fluorescence

Temperature (°C)

\( T_m = 65^\circ \text{C} \)

\( y_U \)

unfolded baseline

\( y_F \)

folded baseline

\( y_U - y_F \)
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.

M. W. Pantoliano et al. (2001) J. Biomol. Screen. 6: 429
D. Matulis et al. (2005) Biochemistry 44: 5258
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 of b-CAII

\[ \Delta T_m \]

\[ [\text{NaCl}] = 0 \text{ M} \]
\[ \text{pH} \text{ 5.8} \]
\[ [\text{NaCl}] = 1 \text{ M} \]

\[ 0.8 \]
\[ 7.0 \]
\[ 10 \]
\[ 20 \]
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, ±MgCl₂.

“Excipient” Characterization:
- Plate-based survey of secondary buffer components:
  - Comparisons of NaCl, KCl, LiCl, NH₄Cl, etc.
  - MgCl₂ vs. MnCl₂ or CaCl₂; different anions (Cl⁻, SO₄²⁻, PO₄³⁻)
  - Cosolutes (amines), polyols (glycerol), surfactants (tween20)
  - Essential elements; NiCl₂, ZnCl₂, etc.

Ligand Binding & Positive Controls:
- Direct measurement of ligand binding affinity (dosed compounds).
- Comparison of binding under different conditions (e.g. ±MgCl₂).

✓ 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
- 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

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

< 90% purity

pure protein →

30% soluble aggregate (1.2mg/ml)

Running buffer
50mM PIPES pH 6.5
100mM NaCl
1mM EDTA
1mM DTT
**Kinase#1: Protein Purification solutions**

- **pH 8.0**
  - higher salt
  - 10% glycerol

- **pH 6.8**
  - lower salt
  - 10% glycerol

- Protein band →

- > 98% purity

**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

-Aggregate

- Monomer

- 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

$pH \pm NaCl \& MgCl_2$

- 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 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).
Enzyme Assay Development: Streamlined Characterization Approach

Challenging System:

- **Substrates**
  - FMNH$_2$
  - FADH$_2$
  - NADPH

- **Products**
  - FMNH$^-$
  - FADH$^-$
  - NADP$^+$

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

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

Condition Profiling
1) pH & Salt
2) excipients

Optimization for Automation
**Enzyme Assay Development:**

Rate-based Product Detection Assays

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1) Fluorometric Dye; Product Chelation

\[ \text{E} + \text{S}_1 \rightarrow \text{E} \cdot \text{S}_1 \rightarrow \text{E} \cdot \text{S}_1^* \cdot \text{S}_2^* \rightarrow \text{E} \cdot \text{P}_2 \]

**Dye•P₂**

Fluorescence Change

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2) Product Capture; Protein Binding

\[ \text{E} + \text{P}_1 \rightarrow \text{E} \cdot \text{P}_1 \rightarrow \text{M} \cdot \text{P}_2 \]

**M•P₂**

Absorbance Change

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**Dye Chelation Assay:**

Variable [Enzyme] - 5 to 1000 nM

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**Secondary Binding Assay:**

Variable [Enzyme] - 5 to 1000 nM
Signal Optimization

Dual Wavelength Absorbance Assay

Abs: Secondary Protein +/- Product

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>340</td>
<td>0.05</td>
</tr>
<tr>
<td>360</td>
<td>0.08</td>
</tr>
<tr>
<td>380</td>
<td>0.1</td>
</tr>
<tr>
<td>400</td>
<td>0.15</td>
</tr>
<tr>
<td>420</td>
<td>0.2</td>
</tr>
<tr>
<td>440</td>
<td>0.25</td>
</tr>
<tr>
<td>460</td>
<td>0.3</td>
</tr>
<tr>
<td>480</td>
<td>0.35</td>
</tr>
<tr>
<td>500</td>
<td>0.4</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Time, minutes</th>
<th>Relative Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.02</td>
</tr>
<tr>
<td>10</td>
<td>0.04</td>
</tr>
<tr>
<td>15</td>
<td>0.06</td>
</tr>
<tr>
<td>20</td>
<td>0.08</td>
</tr>
</tbody>
</table>

0.05 μM enzyme
Conditional Effects on Rx Rates

Array-based Approach:

Survey of pH & Salt:
• The activity rates (after 10min) in the
  range are similar from pH 6.0-7.5.
• Initial rates are similar between 100-
  400 mM NaCl

Excipient effects on Rates:
• Increased Activity Rates:
  CaCl₂, MgCl₂, Tween 20
• Significantly Decreased Rates:
  NiSO₄, PEG, imidazole
• Tween20 optimal at 0.01%; DMSO
  tolerated up to 2%

Buffer modified to HEPES, pH7;
  CaCl₂, MgSO₄, Tween, GSH
  added to minimize [Enzyme].
Initial Automation Uniformity Tests

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

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

*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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