Binding Rate Screen

A High-throughput Assay in Soluble Lysate for Prioritizing Protein Expression Constructs

CHI The Bioprocessing Summit, 2011
2nd Annual ForteBio Workshop
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I. Background
   Need for a novel method

II. The Binding Rate Screen (BRS)
   A. Principle
   B. Platform
   C. Proof of Concept Studies

III. Conclusions
Current Gene-to-Structure Strategy

Protein I: Well-known, difficult-to-crystalize receptor tyrosine kinase (RTK)

Tier 1. KID Scan

<table>
<thead>
<tr>
<th>Tier 1. KID Scan</th>
<th>Tier 2. C- terminus Scan</th>
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<tbody>
<tr>
<td>WT</td>
<td>Best KID Mutants</td>
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<tr>
<td>KID</td>
<td>CT</td>
</tr>
<tr>
<td>N</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td></td>
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<tr>
<td>54</td>
<td>96</td>
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<tr>
<td>KID Mutants</td>
<td>CT Mutants</td>
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<td>at Fixed CT</td>
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Mutants with ΔKID and ΔCT had much LOWER soluble expression than WT or ΔKID
Traditional HTP Purification Strategy

2 × 96 cultures

1. Pellet cells
2. Lyse cells with Lysis buffer
3. Centrifuge and save supernatant
4. Reformat to 96-well plate

5. Filter supernatant
6. Add supernatant to QIAFilter filled with Ni-NTA resin

7. Collect flow through
8. Wash with buffer

Ni-NTA

9. Elute with imidazole buffer

Analyze
1. Soluble lysate
2. IMAC flow through
3. IMAC elution

By
1. SDS-PAGE
2. Dot blot/Western
SDS-PAGE Analysis

**Soluble Expression**

Increasing C-terminus length

| 1 | 2 | 3 | 4 | 5 |

Conclusion:

↑ C-terminus length

↑ soluble expression

**IMAC Elution**

| 1 | 2 | 3 | 4 | 5 |

Flow Through

| 1 | 2 | 3 | 4 | 5 |

- 182 kD
- 115 kD
- 82 kD
- 64 kD
- 49 kD
- 37 kD
Protein in Flow Through is Aggregated

Load FT from IMAC onto Size-exclusion column

- Importance of the quality of the expressed protein
- Klock et al, Proteins 71 (2008) 982-994: Examined proteins post IMAC purification by AnSEC. Scoring system – 1 (aggregated) to 4 (monodisperse). Showed that 3 or 4 is necessary but not sufficient for structure success; 96 samples/day
In-House Experience with a Kinase Campaign

>100 Constructs → Identify Trend → Test Hypothesis → Detailed Study w/ few (6) → Better Understanding → Design Study for selected constructs (20) → Nail the Trend

Construct Selection for Scale-up Purification and Crystallography (10 months… with MANY FTEs)

Lesson Learned

Increasing or improved soluble expression ≠ Quality Protein

Need a Novel HTP Method that can screen for soluble aggregates in crude lysate.
ForteBio: Label-Free Technology

- Sample matrix friendly: crude soluble lysates (CSL), media, serum, etc
- High-throughput: 8 measurements in parallel
- Easy access to fresh sensor surface
- Simple Instrumentation - Sample, Sensors, and Plates
Pilot Experiment: Screen Protein I at Soluble Lysate Stage

Aggregated His-tagged protein does not bind!

Cannot identify soluble aggregates by size.
Idea...

Common reactive-antibody tag

Serving as a probe to sense the microenvironment of the target protein.
Binding Rate Screen (BRS) Principle

Initial Binding Rate ($\nu$) $\propto$ Binding Rate Constant $\times$ Concentration

- Binding Rate Constant: $3 \equiv 2 > 1 > 4$ or $5$
- Faster Binding Rate $\rightarrow$ Desirable Situation

Goals: (1) monodisperse/nonaggregated
(2) sufficient protein yields
(3) biologically active

BRS Ranking:
- : High BR Constant and Conc.
- : High BR Constant and OK Conc ($\sim$1mg/mL).
- : OK BR Constant and Conc.
- : Low BR Constant and high or low conc. High BR Constant and low conc.

- When in doubt, run a SDS-PAGE gel
Protein Samples:

- Lyse cells (*E. coli*, BV, mammalian) with preferred buffer
- Centrifuge; filter if necessary
- Dilute CSL 1:10 ~ 1:100 with Sample Diluent Buffer from ForteBio

Biosensors:

- Generate biotinylated penta-His Ab (Qiagen): Sulfo NHS-LC-LC-Biotin
- Coat sensors following ForteBio procedure
- Can be stored dry: good for at least 6 months

BRS Assay Time:

- Use Quantitation Mode
- ≤ 5 min/ column of samples
Proof of Concept: Protein I

SDS-PAGE Gel

CSL

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<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H&lt;sub&gt;NC&lt;/sub&gt;</th>
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IMAC Elution

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170 kD 110 79 47 35 25 17
**AnSEC Analysis**

**Step 1.**
Load soluble crude lysate onto size exclusion column (SEC).

**Step 2.**
Collect fractions and analyze each by ECL-based MSD technology with anti-His antibodies.

**Step 3.**
Repeat for other constructs.
Within 1 min, can identify the “most promising” construct(s) to pursue

- \( \nu \) value makes prioritizing easy

- Construct E and F both gave crystals; Structure of F (preferred by the crystallographer) was solved.
Binding Rate Constants Differ within Monomeric Protein Variants

Initial Binding Rate ($\nu$) $\propto$ Binding Rate Constant $\times$ Concentration

Constructs with faster $\nu$ have better properties for crystallography.
Other Examples: Protein II (RTK)

- Target Protein from Construct L led to crystals and structure.
Other Examples: Protein II (RTK)

SDS-PAGE Gel (SE)

Constructs:  A  B  C  D  E  F  G  H  I  J  K  L  M  N  O  NC

BRS

ν (initial 10s)
Other Examples: Protein III (DNA Binding Protein)

Construct Design

A ↓ B ↓ C ↓ 6 5 4 3 2 1

N ↓------------------------C

N: 1 2 3 4 * 6
C: 1 2 3 4 5 6

SDS-PAGE

SE

IMAC

BRS

Δλ (nm)

0 70 140 210 280 0 70 140 210 280 0 70 140 210 280 0 70 140 210 280 0 70 140 210 280

22 kD

16

19 | Jiamin Yu | CHI The Bioprocess Summit 2011 | ForteBio Workshop
Other Examples: Protein III (DNA Binding Protein)

BRS

AnSEC

ECL Counts of Monomeric Protein (×1000)

ν (nm/s)

ECL Signal (×10^7)

Elution Fractions

Aggr.
Mono

B1
B2
B3
B4
B5
B6
**Novel functional screen** - uses (His$_6$) tag as a reporter, and measure its binding rate to an affinity matrix as a metric to reflect aggregation, concentration, and purifiability of the target protein.

**Built on ForteBio Platform**
- sample matrix friendly
- high-throughput
- easy to use
- robust instrumentation
- easy & economical access to fresh sensor surface

**Simple, Easy and Fast** - 96 constructs screened in 1 h or less after lysis, saves FTE

**Value Statement**

The value of BRS to structural biology efforts of rapidly generating and characterizing truncations is to prioritize protein variants so that constructs best suited for crystallography can be put into crystal trials first to improve the chances of individual target success.
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**ForteBio**

**diaDexus**
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