Fast, sensitive and selective PTM-peptide immunoprecipitation with AssayMap Bravo

April 23, 2019
Bravo User’s Meeting

Alissa Nelson
Proteomics Scientist
CST Proteomics Group

Support existing PTMScan enrichment kits
- Tech support / validation / lot testing

Motif / PTM Antibody Development

Method Development

Collaborations

Fee-for-Service
PTM and Motif Antibodies Empower PTMScan

Standard
Context-target specific

Motif
Context-dependent

PTM
Context-independent
PTMs
Ser/Thr Phosphorylation
Tyr Phosphorylation
Lysine Acetylation
N-Terminal Acetylation
Ubiquitination
SUMOylation
Mono/Di/Tri-Methyl Lysine
Mono-Methyl Arginine
Asymmetric Di-Methyl Arginine
Symmetric Di-Methyl Arginine
Caspase Cleavage (-DEXD)
Succinylation
Malonylation
Propionylation
Glutarylation
Site Specific (PTM Pathways)
PTMScan Method

Protease-digested Cell Extract

Analyse Eluted Peptide Fraction by LC-MS/MS

Relative Quantification/Data Analysis

Assign Sequences to MS/MS Spectra with SEQUEST®

Protein of Interest

PTM/motif Abs

Cell Signaling Technology
Saving time with a Bravo

Day 1: Lyse & digest proteins
Day 2: Sep-Pak C18 cleanup
Day 3-4: Lyophilize
Day 4: Prepare fresh Ab-agarose beads (15min for overnight incubation)
Day 5: IP motif peptides & desalt
Day 6: 2nd digest (for excess Ab) & 2nd desalt

One day with Bravo:
- Load Ab onto Protein A tips
- IP peptides
- Desalt, 2nd digest, desalt again

12 samples at a time
96 samples at once
Evaluation Timeline

2013: Initial contact with Agilent

Jan 2018: Budget approved!

Spring 2018: Two visits to Lexington, surprised to find poor results

September 2018: Demo Bravo delivered to CST: Older robot method outperformed newer

![Chart showing Unique Motif Peptides with pY and Ubi categories, comparing Manual and Robot methods. The bar for Ubi in the Robot method is significantly higher than that in the Manual method.]
Evaluation Timeline

2013: Initial contact with Agilent

Jan 2018: Budget approved!

Spring 2018: Two visits to Lexington, surprised to find poor results

September 2018: Demo Bravo delivered to CST: Older robot method outperformed newer
Aspirate vs. Dispense Mode

- Old method: both aspirate & dispense
- New method: Dispense only
- New expected to improve reproducibility, prevent clogging in case of particulates
- Aspirate mode promotes better mixing and equilibration
Aspirate vs. Dispense Mode

- Old method: both aspirate & dispense
- New method: Dispense only
- New expected to improve reproducibility, prevent clogging in case of particulates
Reproducibility Experiment Design

- 4 Abs, 4 replicate IP's (robot)
- Manual IP controls
- 100ug Ab per cartridge
- 2mg peptide each
Bravo Reliably Enriches More Motif Peptides than Manual

**AcK**
- Robot: 
- Manual: 
- 47% gain

**Ubi**
- Robot: 
- Manual: 
- 91% gain

**pY**
- Robot: 
- Manual: 
- 135% gain

**PSD Multipathway**
- Robot: 
- Manual: 
- 10% gain

*** p<0.001
ANOVA: Same-day IP’s are just as reproducible as manual

- Analyzing variance in number of unique motif peptide IDs
- Separate prep days > IP replicate tubes/wells > LCMS tech reps
- Manual variance >= Bravo variance
Bravo Quantitative Results : 2x Peak Areas

**pY**

Median = 2.0 (linear)

**Ub**

Median = 2.5 (linear)

**AcK**

Median = 2.8 (linear)

**MP**

Median = 1.4 (linear)
PTMScan for Client Projects: How many cartridges?

- Manual SOP: 15mg peptide per IP, Development experiments all done with 2mg peptide/IP
- Criteria: Equal or better # of motif identifications compared to current SOP
- Limitation: 250uL peptide load volume
- Max peptide load onto robot protein A tips??

<table>
<thead>
<tr>
<th>Peptide: Ab ratio</th>
<th>ug Ab</th>
<th>Mg Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>60</td>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td>40</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Load more peptide per tip?... x n tips

= N mg
Selectivity behavior differs in manual vs Bravo

- Max peptide solubility: 16mg/mL $\rightarrow$ 4mg per 250uL load
- Constant bead volumes, constant Ab
- Manual: Unmodified peptides are constant regardless of peptide input; bead volume may be the determining factor
# Scale-up: 1-4 tips x 4mg

<table>
<thead>
<tr>
<th>Ab</th>
<th>Tissue</th>
<th>MS</th>
<th>IP</th>
<th>Desalt</th>
<th>2nd Digest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubi</td>
<td>Liver</td>
<td>Lumos</td>
<td>Manual 16mg</td>
<td>Manual C18</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Robot 4mg, 8mg, 12mg, 16mg</td>
<td>Manual C18 (single tip)</td>
<td></td>
</tr>
<tr>
<td>Ubi</td>
<td>Muscle</td>
<td>QE</td>
<td>Manual 16mg</td>
<td>Manual C18</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Robot 4mg, 8mg, 12mg, 16mg</td>
<td>Robot C18, 1/2/3/4 tips</td>
<td></td>
</tr>
<tr>
<td>pY</td>
<td>Embryo</td>
<td>QE</td>
<td>Manual 16mg</td>
<td>Manual C18</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Robot 4mg, 8mg, 12mg, 16mg</td>
<td>Robot C18, 1/2/3/4 tips</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Robot 4mg, 8mg, 12mg, 16mg</td>
<td>Robot C18, 1/2/3/4 tips</td>
<td>Manual</td>
</tr>
</tbody>
</table>
Peptide input reduced to 25% of manual SOP

One tip (4mg) is sufficient for Ubi, gives up less than 10% for Ack and pY

- pY embryo QE
- Ubi muscle QE
- Ac-K liver QE
- Ubi liver Lumos
Robot enhances selectivity

- pY sees nearly double the selectivity with one 4mg robot tip vs SOP 16mg
- All IPs improve by 5-18 percentage points
Client Study: pY x 24 conditions

- Major pharma client
- 4 cell lines (“blue”, “orange”, “green”, “red”)
- Drug candidate treatment vs control @ 3 timepoints (=24 total samples)
- Four enrichments:
  - pY
  - mme K/R
  - Phospho motif mix
  - AcK
- 12+ days of benchwork for enrichments alone cut down to 4
Conclusions/Future Directions

- AssayMap Bravo outperforms manual enrichment of samples
  - More modified peptides identified
  - Higher relative peptide abundance
  - Better reproducibility qualitative + quantitative

- Aspirate-mode protocol critical for performance

- Validate other protocols (Fe-IMAC, digests for total proteome)

- **PTM/Motif Antibodies available off-bead for robotic applications**
Rooted in Science. Grounded in Reproducibility.℠

**Cell Signaling Technology**
Matt Stokes
Charles Farnsworth
Matthew Fry
Xiaoying Jia
Yijing Zhu
Jian Min Ren
Vicky Yang
Kimberly Lee
Roberto Polakiewicz

**Agilent**
Steve Murphy
Steve Lozano
Martin Thomae