Proteomics Standards Research Group (sPRG)

www.abrf.org/sprg

ABRF-sPRG09: Development of a Quantitative Proteomics Standards
sPRG 2009 Study Goal

- A tube containing sets of Natural and Labeled Peptides in defined ratios and amounts.
- Natural peptides: Derived from trypsin digest of several proteins found in human plasma.
- Stable Isotope Labeled peptides: differing only in mass from the Natural peptides
- Suitable for use in assessing a laboratory’s capabilities for absolute quantitative analysis.
Synthesize SIL Peptides -> Purify SIL Peptides -> Subaliquot & Freeze All SIL Peptides -> Quantify -> Select SIL: Protein Ratios

Subaliquot, Dry & Mail Samples

Add SIL Peptides into Digest of Proteins

Grow Bugs; Isolate Proteins -> Purify Proteins -> Subaliquot & Freeze All rProteins -> Quantify -> Digest Proteins; Mix Tryptic Peptides
sPRG2009 Study

- One tube containing purified recombinant proteins in specified amounts which were then:
  - Reduced, Alkylated and Trypsin Digested and
  - Spiked with selected Stable Isotope Labeled peptides in specified amounts corresponding to natural trypsin peptides.
  - Aliquotted, dried and sent to requesters.
- Requesters determine the relative ratios of Natural to Heavy peptides
- Sufficient material will be provided so that participants can perform a number of replicate analyses.
- Participants will be invited to use the LC-MS proteomics workflow of choice to determine the absolute concentration of constituent proteins.
- Participants will be able to report their results via a web-based survey tool.
sPRG2009 Study

• Proteins:
  • Recombinant human proteins
  • Derived from the constituents found in Sigma’s Universal Proteomics Standard
  • Based on the sPRG2006 Study sample
Tryptic Peptides Corresponding to the Proteins

- At least 3 Peptides per protein
- Meeting a Number of Criteria
Peptide Considerations: Synthetic & Natural

- Length: $8 < N < 20$ amino acid residues
- Avoid Possible Imide Sites: [DN].[GASN]
- Avoid DKP Formation: C-terminal peptide with G or P at C2 position.
- Avoid Multiple Amino Acid Repeats: e.g., XXX
- Avoid Overly Hydrophobic Sequences: multiple aliphatic aa’s e.g., A, L, I, V
- Minimize Incomplete Side Chain Removal: tBu and Trt
Peptide Considerations: Natural & Synthetic

• No Acid Labile Residues: [DP]
• No Oxidation Sensitive Residues: Met, Trpₙ
• No N-terminal Gln Residues (→pyroGlu)
• No Pro-Pro; cis-trans isomers in chromatography

Chromatography:
• Retained by C18 column and
• Eluted between 2-75% MeCN buffer
Peptide Considerations: Natural

• Enzymatic Digestion: Has to Be Very Good
  • Every Missed Cleavage Affects the Quantitation
  • Every Semi-Tryptic Cleavage Affects the Quantitation

• No N- or C-terminal KK, KR, RK, or RR cleavages; no KP or RP cleavages

• No known PTMs or consensus recognition sequences (e.g., N-glycosylation)

• Chromatography: Peptides retained by C18 column and eluted in a reasonable percentage of MeCN buffer
Real World Consideration

• If you want to detect the protein, you’ve got to pick a peptide or two…
• For our study: Minimum of 3 proteotypic peptides per protein
Testing Pilot Sample

- 5 Proteins: Digested Individually then pooled in 3 different amounts
- Spiked with corresponding SIL peptides all at the same level
- Dried and sent to sPRG members to test for ratios
Results for Pilot Study

• Ratios off!
• Back to Drawing Board with Clock Ticking
Plan for Prototype 1

• Test Four Digest Conditions
  • Pick best
• Repurify Proteins
  • Subaliquot and Store at -80C until used.
• Quantitate Proteins and SIL Peptides:
  • AAA in triplicate
• Perform Digest on Protein Pool and Test
• Formulate Digest with SIL Peptides
• Test Prototype 1 in sPRG Labs
Quantitation

• Amino Acid Analyses (AAA)
• Proteins and SIL peptides
• In Triplicate
• In Two Labs
Testing of Digest Conditions

• Protein Mixture (based on BCA analysis):
  • ALBU: 140 pmol
  • SYHC: 88 pmol
  • NQO2: 70 pmol
  • PRDX1: 88 pmol
  • UBIQ: 147 pmol

• Instrumentation: Michrom BioResources Paradigm MS4 HPLC; Thermo Fisher LTQ-FT. MS analyses: data-dependent acquisition of the eight most abundant ions in the survey scan; precursors were detected in the FT, fragmentation was conducted in the LTQ.

• Data analyses: Mascot database search against the Swiss-Prot database (human) using 10-ppm mass accuracy for precursor ions and 0.5-Da accuracy for product ions; Scaffold (Proteome Software) was used for processing of the Mascot search results.
# Testing of Digest Conditions

<table>
<thead>
<tr>
<th>Method</th>
<th>ALBU</th>
<th>SYHC</th>
<th>NQO2</th>
<th>PRDX1</th>
<th>UBIQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin, no denaturant</td>
<td>518 (100)</td>
<td>230 (48)</td>
<td>161 (36)</td>
<td>171 (30)</td>
<td>30 (6)</td>
</tr>
<tr>
<td>Trypsin/Lys-C, urea (8 M)</td>
<td>318 (68)</td>
<td>147 (39)</td>
<td>101 (25)</td>
<td>110 (27)</td>
<td>23 (8)</td>
</tr>
<tr>
<td>Trypsin, TFE (40%)</td>
<td>375 (77)</td>
<td>132 (33)</td>
<td>92 (25)</td>
<td>107 (19)</td>
<td>62 (9)</td>
</tr>
<tr>
<td>Trypsin, urea (6 M)</td>
<td>401 (75)</td>
<td>155 (42)</td>
<td>102 (25)</td>
<td>112 (24)</td>
<td>34 (8)</td>
</tr>
</tbody>
</table>
The Winning Digest Protocol

• Reconstitute the sample in 20 uL of 2 mM TCEP, 50 mM NH₄HCO₃, 1 mM CaCl₂, pH 8.6 buffer.
• 15 minutes at 60°C in the shaker. Cool to 20°C. Add 5 uL of the iodoacetamide stock. 30 min @ 20°C & shaking/dark.
• Add 4.5 uL of the TCEP stock to the tube. 30 min @ 37°C & shaking/dark.
• Add 18 uL of H₂O, and 4 uL of the reconstituted trypsin (15:1 S:E by weight). 16 hrs @ 37°C in the shaker.
• Add another 4 uL of the reconstituted trypsin 6 hrs @ 37°C in the shaker. Final volume is ~55 uL, 20 mM AmBic buffer, 5 mM TCEP-acetamide, < 0.5 mM unreacted TCEP.
• Bring to the volume to 83.3 uL by adding 28.3 uL of 1% formic acid.
Prototype 1 Preparation

- Amounts of Protein Added was based on AAA concentrations.
  - Quantities: from 10 pmol to 0.1 pmol per tube.
- Digested with “Trypsin, no denaturant” protocol.
- Analyses of digests of the five proteins provided insight into the tryptic peptides that were most reliably detected.
## Proteins in Prototype-1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass (kDa)</th>
<th>2+ (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Albumin (ALBU_HUMAN) , 66.4 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K.DLGEEFK.A</td>
<td>476.2</td>
<td>480.2</td>
</tr>
<tr>
<td>K.LVNEVTEAK.T</td>
<td>575.3</td>
<td>579.3</td>
</tr>
<tr>
<td>K.SLHTLGDK.L</td>
<td>509.3</td>
<td>513.3</td>
</tr>
<tr>
<td>K.AEFAEVSK.L</td>
<td>440.3</td>
<td>444.7</td>
</tr>
<tr>
<td>Histidyl-tRNA synthetase (SYHC_HUMAN) , 57.4 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K.DQQGEELSLR.Y</td>
<td>544.3</td>
<td>549.3</td>
</tr>
<tr>
<td>K.GLAPEVADR.I</td>
<td>464.2</td>
<td>469.3</td>
</tr>
<tr>
<td>R.AALEELVK.L</td>
<td>436.8</td>
<td>440.8</td>
</tr>
<tr>
<td>R.IFSIVEQRL</td>
<td>496.3</td>
<td>501.3</td>
</tr>
<tr>
<td>Ribosylhydronicotinamide dehydrogenase (NQO2_HUMAN) , 25.8 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K.NVAVDELSR.Q</td>
<td>501.8</td>
<td>506.8</td>
</tr>
<tr>
<td>R.SLASDITDEQK.K</td>
<td>603.8</td>
<td>607.8</td>
</tr>
<tr>
<td>R.EADLVI FQFPLYWFPSVAILK.G</td>
<td>1248.7</td>
<td>1252.7</td>
</tr>
<tr>
<td>Peroxiredoxin-1 (PRDX1_HUMAN) 22.1 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K.DISLSDYK.G</td>
<td>470.7</td>
<td>474.7</td>
</tr>
<tr>
<td>K.ADEGISFR.G</td>
<td>447.7</td>
<td>452.7</td>
</tr>
<tr>
<td>R.GLFIIDDG.G</td>
<td>460.8</td>
<td>464.8</td>
</tr>
<tr>
<td>R.LVQAQFTDK.H</td>
<td>598.8</td>
<td>602.8</td>
</tr>
<tr>
<td>Ubiquitin (UBIQ_HUMAN) 9.4 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R.TLSDYNIQK.E</td>
<td>541.3</td>
<td>545.3</td>
</tr>
<tr>
<td>K.ESTLHLVLR.L</td>
<td>534.3</td>
<td>539.3</td>
</tr>
<tr>
<td>K.TITLEVEPSDIENVK.A</td>
<td>894.5</td>
<td>898.5</td>
</tr>
</tbody>
</table>
Prototype 1 Preparation

- Added corresponding SIL peptides to achieve a 1:1 ratio with the original concentration of each protein in the mixture.
- Aliquotted into vials and dry by vacuum centrifugation.
- Sent to sPRG labs for testing.
Conclusions

• Quantitative Analysis of Proteins Using SIL peptides:
  • Optimize the digestion protocol to achieve maximum sequence coverage and reproducibility
  • Select multiple representative proteolytic peptides that are reproducibly generated for each protein
  • Validate the purity/quantity of the SIL peptides

• Analysis of Prototype-1, the sPRG is now ready to prepare and distribute the 2009 study sample to requestors.

• Presentation of results at the 2009 ASMS.
Acknowledgements

• AAA: Barrett Smith (UC Davis)
• QQQ analyses: Rudy Alvarado (UC Davis);
• Data Interpretation: Johann Holzmann (Research Institute of Molecular Pathology, Vienna)
• HPLC-ESI-MS/MS: Kevin Hakala (UTHSCSA)
• Former Chair: Rachel Ogorzalek Loo (UCLA)
Acknowledgements

• Invaluable help was also provided by the following people at Sigma Life Sciences:
  • Henry Duewel
  • Shantanu Roychowdhury
  • James Walters
  • Kevin Ray
  • Kristin Rolwes
  • George Lipscomb.
Proteomics Standards Research Group

- Dave Arnott, Genentech, Inc.,
- Jim Farmar, Einstein College of Medicine
- Alexander Ivanov, Proteomics Resource, Harvard School of Public Health
- Jeff Kowalak, National Institute of Mental Health
- William Lane, Harvard University FAS Center for Systems Biology
- Karl Mechtler, Research Institute of Molecular Pathology, Vienna
- Brett Phinney, Proteomics Core UC Davis Genome Center
- Manfred Raida, Experimental Therapeutics Centre, SG
- Sue Weintraub, Univ. of Texas Health Science Center
For More Details, Please visit our Poster:

- sPRG Poster RG5 S1
- “ABRF-sPRG09: Development of a Quantitative Proteomics Standards”