Introduction to Proteomic Sample Preparation for Mass Spectrometry
Two Main Approaches to Proteomics

**Bottom Up Proteomics**

1. Proteins
2. Proteolytic Digestion
3. Peptides
4. HPLC or SPE
6. Analysis

**Top Down Proteomics**

1. Proteins
2. HPLC, Gelfree, CE
4. Analysis

adapted from http://www.piercenet.com/method/sample-preparation-mass-spectrometry
Sample preparation is one of the most important aspects of any successful proteomics experiment!

Questions you should ask before proteomics sample prep:

1. How should I collect the samples?
   - Consistency, consistency…
   - ...and more consistency
   - Sampling for biological variation
   - Document all steps from sample collection thru prep – Good lab notes!

2. What do I hope to analyze?
   - Simple protein ID
   - Differentially expressed proteins (ITRAQ, SILAC, label free or targeted)
   - Post-translational modifications (PTM)
   - Cytoplasmic, membrane, nuclear, mitochondrial, etc.
   - Immunoprecipitation/binding partners
Questions before starting prep (continued):

3. How do I best extract my proteins of interest?

   In-gel: SDS-PAGE (1D or 2D)
   In-solution
   Enrichment/affinity strategies
   Depletion of high abundant proteins

4. Is the extraction buffer compatible with mass spectrometry? If not, how do I get rid of problematic buffer components?

   Solid Phase Extraction (SPE), detergent removal spin-column
   Cloud point extraction of non-ionic detergents
   Ethyl acetate extraction of detergents
   Protein precipitation
   Dialysis, molecular weight cut-off spin filter
Overview: Sample Prep to Mass Spectrometry Work Flow

**Solid Tissue and cell culture samples**

- Disruption:
  - Sonication
  - Bead beater/homogenizers
  - Pressure cycling technology
  - Freeze + mortar & pestle
  - Enzymatic
  - Heat, Freeze/thaw

- Extraction Buffer (denature proteins):
  - Chaotropes (Disorder maker)
    - Urea, thiourea, guanidine HCl, salts (KCl, NaCl, etc.), MeOH, acetonitrile
  - Detergents
    - Ionic (SDS), nonionic (NP40, triton x-100), zwitterionic (CHAPS)
  - Buffer reagent – pH considerations
    - Tris, HEPES, ammonium bicarbonate, PBS, TEAB
  - Reducing reagents
    - Dithiothreitol (DTT), TCEP (tris(2-carboxyethyl)phosphine)

**Biological Fluids, Media, other solution preps (i.e. IP’s)**

- MW Spin filter
- Dialysis
- Precipitation
- Denaturing Buffer

Common Extraction Buffers:
- 4% SDS, 100mM Tris pH 7.6, 100mM DTT
- 7M urea, 2M thiourea, 0.4M TEAB pH8.5, 20% acetonitrile, 4mM TCEP

Centrifuge insoluble material out & aspirate supernatant (protein extract)

Depletion of high abundant proteins typically done before digestion

Enrichment of post-translational modifications typically done after digestion
Disruption with the Barocycler®
NEP2320
Maximizing Protein Extraction from Tissue

Pressure Cycling Technology Sample Preparation System
(PCT SPS)
...the Power of PCT

- Pressure Cycling Technology (PCT) uses cycles of hydrostatic pressure between ambient and ultra high levels allowing for a high degree of speed, reproducibility, and convenience.

- 1 PSI input produces 440 PSI output resulting in a top pressure of 35 kPSI.

- Better extraction from various sample types and quicker enzymatic treatments (proteolytic cleavage, deglycosylation, etc.)
Pressure Compresses Lipids Beyond Equilibrium

Hydrostatic Pressure Applied

Rapid De-pressurization Causes Membranes and Micelles to Disintegrate

Hydrostatic Pressure Rapidly Released

PCT = pressure cycling technology, 10 cycles, 20 sec. 35 kpsi, 20 sec ambient press.

GG = ground glass dounce homogenizer

500 ug loaded on each gel

<table>
<thead>
<tr>
<th>Method</th>
<th>Spots detected</th>
</tr>
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<tbody>
<tr>
<td>PCT</td>
<td>2280 ± 173</td>
</tr>
<tr>
<td>GG</td>
<td>1620 ± 137</td>
</tr>
<tr>
<td>GG/sonication</td>
<td>1735 ± 144</td>
</tr>
<tr>
<td>GG/sonication x 2</td>
<td>1682 ± 165</td>
</tr>
</tbody>
</table>

Values are mean ± SD (n = 5).
# -/+ Barocycler Prep & Digestion

## mouse liver tissue

<table>
<thead>
<tr>
<th>Treatment</th>
<th><strong>ABI 4800</strong></th>
<th><strong>Thermo LTQ</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Protein ID’s</strong></td>
<td><strong>Protein ID’s</strong></td>
</tr>
</tbody>
</table>
| **Std. Lysis/Std. Digest** | 115 (99\%Protein Pilot)  
203 (95\%Protein Pilot) | 118 (≥3peptides 99\%, Scaffold) |
| **Std. Lysis/PCT Digest** | NR | 219 |
| **PCT Lysis/Std. Digest** | 275  
471 | 264 |

PCT Lysis = 30 cycles @ 37\(^\circ\)C, 50sec.  
35kpsi, 10sec. Ambient press.  

PCT Digest = 60 cycles @ 37\(^\circ\)C,  
50sec. 20kpsi, 10sec. Ambient press.  

Std. Lysis = mortar & pestle + sonication  
Std. Digest = overnight(16hrs.)  

Lysis Buffer = 8M urea, 0.2\%SDS, 0.5M TEAB, 5mM TCEP  
Std. Digest = reduce, alkylate, dilute sample to 2M urea, add trypsin in 1:25 ratio, incubate overnight @ 37\(^\circ\)C
Biological Fluid Prep Example - Urine

1. Urine Sample
2. MW Cut-off Spin Filter
3. Recover sample
4. Dialyze
5. Vacuum Centrifuge to Dryness
6. Resuspend with Solubilization buffer
7. Protein Concentration Assay (Bradford or BCA)
8. Proteolytic Digestion
9. HPLC or SPE
Two main sample prep paths to take...choose wisely!

“When You Come to a Fork in the Road, Take It!” –Yogi Berra

Gel based strategy
2D gel or 1D gel

Non-Gel based strategy
Protein + Trypsin = Peptides

ISD or FASP

LC/LC-MS

In-gel Proteolytic Digestion

Washing: A series of water, ammonium bicarbonate and acetonitrile additions and aspirations to remove impurities from the separation

Reduction: DTT or TCEP + heat

Alkylation: iodoacetamide (+57 mass shift on Cys) methyl methanethiosulfonate (+46 on Cys)

Washing and Dry: ammonium bicarb./acetonitrile washes dry with 100% acetonitrile – no speedvac

Digestion: Trypsin 5 ng/ul, 25-100mM ammonium bicarbonate other enzymes, AspN, GluC, Chymotrypsin

Incubate trypsin digest overnight @ 37°C

Extraction of peptides: 50/50 acetonitrile/water, 0.1% TFA or FA
75/25 acetonitrile/water, 0.1% TFA or FA pool digest and extracts for same sample dry down in vacuum centrifuge for SPE or mass spec.
In-Solution Proteolytic Digestion

Reduction: DTT or TCEP + heat

Alkylation: iodoacetamide (+57 mass shift on Cys) methyl methanethiosulfonate (+46 on Cys)

Dilute buffer components that interfere with proteolytic enzyme: urea < 2M, thiourea < 1M SDS < 0.1%

Digest: Add trypsin (proteolytic enzyme) in 1:35 to 1:100 enzyme to total protein ratio

Incubate overnight (12-16hrs) @ 37°C

Freeze digest at -80°C and dry down for SPE of peptides

*Consider if buffer components can be cleaned up with SPE or HPLC step before mass spec., otherwise need to rethink buffer

Adapted from: www.cshprotocols.cshlp.org
Filter Aided Sample Prep - FASP

a  Lysate, SDS and DTT
Add 8 M urea
Centrifuge (remove SDS, DTT and low-molecular-weight material)

b  Retenate
Add 8 M urea and IAA
Incubate
Centrifuge (remove excess IAA)

c  Retenate
Add 8 M urea
Centrifuge (remove any remaining reagent)

d  Retenate
Add endoproteinase
Incubate
Centrifuge to collect peptides (eluate)


Adapted from www.piercenet.com
<table>
<thead>
<tr>
<th>Protease</th>
<th>Cleavage site</th>
<th>Example of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys-C Specific protease</td>
<td><strong>NNNNK</strong>&lt;br&gt;<strong>NNN (K is lysine)</strong></td>
<td>Digests membrane and other proteolytically resistant proteins; generates larger peptides than tryptic peptides—advantage for certain mass spec methods (for example, ETD)</td>
</tr>
<tr>
<td>Arg-C Specific protease</td>
<td><strong>NNNNR</strong>&lt;br&gt;<strong>NNN (R is arginine)</strong>&lt;br&gt;<strong>Arg-C can, at a lesser degree, cleave at lysine also</strong></td>
<td>Facilitates analysis of histone posttranslational modifications; used in proteome-wide analysis</td>
</tr>
<tr>
<td>Glu-C Specific protease</td>
<td><strong>NNNNE</strong>&lt;br&gt;<strong>NNN (E is glutamate)</strong>&lt;br&gt;<strong>Glu-C can, at a lesser degree, cleave at aspartate residue also</strong></td>
<td>Used as an alternative to trypsin if trypsin produces too short or too long peptides or if tryptic cleavage sites are not accessible</td>
</tr>
<tr>
<td>Asp-N Specific protease</td>
<td><strong>NNNND</strong>&lt;br&gt;<strong>NNN (D is aspartate)</strong></td>
<td>Similar to Glu-C</td>
</tr>
<tr>
<td>Chymotrypsin Low Specific protease</td>
<td><strong>NNNN(F/Y/W)</strong>&lt;br&gt;<strong>NNN (F, Y and W are aromatic residues phenylalanine, tyrosine and tryptophan)</strong></td>
<td>Digests hydrophobic proteins (for example, membrane proteins)</td>
</tr>
<tr>
<td>Pepsin Nonspecific protease</td>
<td>Nonspecific protease (advantage—most active at low pH)</td>
<td>Used in structural protein studies and antibody analysis; digests proteolytically resistant, tightly folded proteins</td>
</tr>
<tr>
<td>Thermolysin Nonspecific protease</td>
<td>Nonspecific protease (advantage—remains active at high temperature)</td>
<td>Digests proteolytically difficult, tightly folded proteins; used in structural protein studies</td>
</tr>
<tr>
<td>Elastase Nonspecific protease</td>
<td>Nonspecific protease</td>
<td>Used to increase protein coverage</td>
</tr>
</tbody>
</table>

www.promega.com
Solid Phase Extraction (SPE)/Clean-up of Peptides

Peptides + Digest Buffers

- Only Buffer & Salts
- C18 material
- MCX - Mixed Mode Cation Exchange
- Sodium Dodecyl Sulfate (SDS)
- other anionic hydrophobic contaminants
- Nonionic/Zwitterionic/ionic Detergents (triton-x100, CHAPS, NP40, SDS, etc.)

Detergent removal spin columns

- Cloud Point Extraction – only for nonionic detergents
  (J. Proteome Res., 2010, 9 (8), pp 3903–3911)
- Ethyl acetate precipitation of detergents

Cartridge images: www.perkinelmer.com
tips & Empore image: www.sigmaaldrich.com
C18 resin image: www.lamondlab.com

image: biotage.phosdev.se

Pierce detergent removal: http://www.fishersci.com
Lysate Preparation/Protein Extraction

Solid Tissue and cell culture samples

- sonication
- bead beater/homogenizers
- pressure cycling technology
- freeze + mortar & pestle
- Enzymatic
- Heat, Freeze/thaw

Extraction Buffer (denature proteins)

- Disruption

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- Centrifuge insoluble material out & aspirate supernatant (protein extract)

Depletion of high abundant proteins typically done before digestion

Enrichment of post-translational modifications typically done after digestion

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Dynamic Range Problem In Samples – A Case for Depletion


Slide courtesy Bruno Domen, ETH Zurich
Beckman Coulter IgY-12 Protein Partitioning Column


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Current Agilent Multiple Affinity Removal System (MARS)

- Anti-Albumin-resin
- Anti-Transferrin-resin
- Anti-Haptoglobin-resin
- Anti-α-1-antitrypsin-resin
- Anti-IgA-resin
- Anti-IgG-resin

Apply Crude Human Serum

Low-Abundant Proteins Free from Interferences

Individual Ab materials are mixed in selected percentages and packed into a column format. Cost of the product is driven by cost of the antigen.

Reference: Agilent web seminar slides
Sigma-Aldrich Human IgY14 and SuperMix Columns

1DE of IgY 14/SuperMix Column Samples

M: MW Marker
P: Plasma
F1: Flow-Through of IgY 14
E: Bound/Eluted Fraction from SuperMix
F2: Flow-Through of IgY 14 + SuperMix
4-20% SDS-PAGE under reducing conditions

4μg Protein loaded per lane


Enrichment - Post Translational Modifications (PTM’s)

Why Enrich for PTM of interest?
In most cases, stoichiometric ratio of PTM species to unmodified species is extremely low.

Jensen Nature Reviews Molecular Cell Biology 7, 391-403 (June 2006) | doi:10.1038/nrm1939
Phosphoproteomic Enrichment

PhosphoSerine
\[ C_6H_9NPO_5 \]

PhosphoThreonine
\[ C_4H_8NPO_5 \]

PhosphoTyrosine
\[ C_9H_10NPO_5 \]

Reference: TE Thingholm, Proteomics 2009, 9, 1451–1468

http://www.ionsource.com/Card/phos/phos.htm
Acetylation Enrichment with Anti-Acetyl Lysine Antibody

IP Method Considerations

Reference: piercenet.com
In-Solution vs. In-Gel Strategies – Dynamic Range

In-Solution Prep, 11 proteins

<table>
<thead>
<tr>
<th>#</th>
<th>Bio View</th>
<th>Accession Number</th>
<th>Molecular Weight</th>
<th>Protein Grouping Abundance</th>
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<tbody>
<tr>
<td>1</td>
<td>Serum albumin precursor (Allergen Bos d 6) (8SA) CRAP</td>
<td>gi</td>
<td>1351907...</td>
<td>69 kDa</td>
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<td>alpha-2-HS-glycoprotein preproprotein [Homo sapiens]</td>
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<tr>
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<td>alpha-fetoprotein precursor [Homo sapiens]</td>
<td>gi</td>
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<td>69 kDa</td>
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In-Gel Prep, 401 proteins

<table>
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<tr>
<th>#</th>
<th>Bio View</th>
<th>Accession Number</th>
<th>Molecular Weight</th>
<th>Protein Grouping Abundance</th>
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<tr>
<td>1</td>
<td>Serum albumin precursor (Allergen Bos d 6) (8SA) CRAP</td>
<td>gi</td>
<td>1351907...</td>
<td>69 kDa</td>
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<tr>
<td>2</td>
<td>Alpha 2-macroglobulin OS=Bos taurus G...</td>
<td>Q5SH1H</td>
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<td>Complement C3 OS=Bos taurus G...</td>
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</tbody>
</table>

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INTRO: Protein Quantitation Strategies at the Peptide Level by Mass Spectrometry†

- **Discovery-based** (complex mixture)
  - Stable isotope incorporation
    - iTRAQ® *
    - SILAC **
  - ‘Label free’
    - Spectral counting
    - Peptide Ion Peak Intensity: XIC-based (extracted ion chromatogram)

- **Targeted analyses**
  - Select peptides of interest
  - Internal standard for absolute quantitation

† Instrument-specific capabilities required
* iTRAQ® Isobaric Tag for Relative and Absolute Quantitation
** SILAC Stable isotope labeling with amino acids in cell culture
Isobaric Tags for Relative & Absolute Quantitation (iTRAQ®)

Example: Compare Relative Protein Expression Levels in Healthy vs Disease Tissues

Experimental Design for 4-plex iTRAQ® Experiment

1. Obtain protein-containing sample, extract protein

2. Proteolytic Digestion
   - Reduce, alkylate Cysteines
   - Trypsin Digest

3. Label peptides with iTRAQ® Reagents
   - iTRAQ TAG 114
   - iTRAQ TAG 115
   - iTRAQ TAG 116
   - iTRAQ TAG 117

4. MIX

5. 2D LC-MS/MS

2D Liquid Chromatography-MS/MS

Try to break down sample complexity

1st Dimension offline HPLC
High pH C18 RP

2nd Dimension HPLC
Inline w/Mass Spec.
Low pH C18 RP

iTRAQ® 8-Plex Reagent Chemical Structure

Isobaric Tag
Total mass = 305

Reporter Group
113 – 119, 121 m/z

Balance Group (?)
Mass 184, 186 – 192 m/z

Amine specific peptide reactive group (NHS)
N-hydroxysuccinimide

This will react with primary amines...need to ensure buffer components are compatible or cleaned-up!

Applied Biosystems has granted permission to use this slide.
SILAC Metabolic Labeling Experimental Workflow

http://www.piercenet.com/method/quantitative-proteomics
SILAC Experiment: Proteome Dynamics of *B. subtilis* in Response to Two Nutritional Challenges

- **growth on succinate**
- **phosphate starvation**

- "normal AA" Lys-$^{12}$C$_6$$^{14}$N$_2$
- "heavy AA" (+8Da) Lys-$^{13}$C$_6$$^{15}$N$_2$

Treated cells (succinate or low P) → Control cells → SILAC incorporation check

Lyse and Combine 1:1

GeLC-MS (12 slices: S1, S2, P1, P2)

Proteolysis (Lys-C)

- Off-Gel 1 (12 wells; S1, S2, P1, P2)
- Off-Gel 2 (12 wells; S1, S2, P1, P2)

SCX → TiO$_2$ → nanoLC-MS/MS

adapted from Soufi B et al; J. Proteome Res. 2010, 9, 3638-3646. Copyright 2010 ACS
Label Free and Targeted Quantification Prep

Label-free Quantitation

Targeted

Metabolic Labeling

Spiked Heavy Peptides

adapted from http://www.piercenet.com/method/quantitative-proteomics