Credentialing Plasma and Serum Biospecimen Banks for Proteomics Analyses

Katy Williams, Ph.D.
Assistant Professor
Dept. of Obstetrics, Gynecology, and Reproductive Sciences
Sander-Moore Mass Spectrometry Core Facility
University of California San Francisco
Mass Spectrometry-Based Proteomics Analyses

• Protein Identification
  – Posttranslational modifications

• Protein Quantification
  – Relative Quantitation
    • Biomarker Discovery
      – Disease specific expression levels
      – Early detection, molecular classification, and diagnosis
    • Protein Interactions
  – Absolute Quantitation
    • Biomarker Verification
    • Protein Modifications
Protein Integrity for Proteomics Analyses

- Proteolysis
- PTMs
  - Phosphorylation
  - Glycosylation
  - Oxidation
  - Nitration
  - Acetylation
  - Methylation
  - Acylation
  - Sulfation
- Aggregation and precipitation
Plasma and serum for biomarker studies

• Protein and peptide integrity in serum and plasma can be compromised in multiple ways
  – Artifactual degradation can be a confounding factor in biomarker discovery experiments

• Determine the effects of specific pre-analytical variables on biospecimen integrity
  – Assess the impact of preanalytical variables at a global level
  – Sample quality sufficient to yield reproducible, high-quality data

• Define quality assessment measures for biospecimens used in proteomics workflows
  – Sensitive markers that can be used as a QC tool to monitor preanalytical variation
Strategy

• Generate a defined set of serum and plasma collected and processed under well defined, well controlled conditions
  – Blood collection using CPTAC protocol
  – Processing using CPTAC (plasma) and EDRN (serum) SOPs
  – Training set
    • Optimally processed samples
    • Processing and storage variables: time, temperature, freeze-thaw cycles

• Establish ranges for candidate markers of protein damage
  – Values observed for optimally processed specimens vs. variables
  – Measure in banked plasma and serum samples

• Establish a panel of reference markers
Pre-analytical Variables in Blood Collection and Processing

- **Inter-individual and intra-individual variation**
  - Age, gender, history, genetics

- **Venipuncture**
  - Needle gauge, butterfly needle, tubing, adapter type

- **Phlebotomy**
  - Tourniquet technique
  - Patient position, arm position
  - Tube order - first vs last, discard tube

- **Collection device**
  - Gel or non-gel separator tube
  - Tube additives, e.g. anti-coagulants or clot activator
  - Manufacturer & device information
  - Tube temperature

- **Blood processing**
  - Time and temperature prior to centrifugation
  - Centrifugation: speed, duration, temperature
  - Protocol for separation of blood from cells
  - Length of time before freezing

- **Storage**
  - Frozen before analysis: snap-frozen, slowly cooled
  - Storage temperature
  - Storage time prior to analysis
  - Number of freeze/thaw cycles
Blood Processing Workflow

CPTAC phlebotomy

- Plasma tubes
  - Ice <30'
  - RT 2''
  - RT 6''

- Serum tubes
  - RT 45'
  - 4C 4''
  - RT 4''

All tubes are processed and stored at -80 °C:
- P1
- P2
- P3
- S1
- S2
- S3

Freeze/thaw:
- 1x
- 2x
Quantitative Measures of Protein Integrity

• MS-based proteomics strategy
  – Identify products of ex vivo proteolytic degradation

• ELISA assays
  – Quantify the levels of protein oxidation and nitration modifications

• Size Exclusion Chromatography
  – Quantify the extent of sample aggregation

Comparison to optimally processed samples
Quantitative Measure of Proteolysis
iTRAQ Labeling Workflow

- Multiplexed assay: 8 samples
- Peptide identification
- Relative quantitation
- Unbiased, non-targeted strategy
Protein Oxidation and Nitration

• Oxidative stress can result in the formation of reactive oxygen and nitrogen species
  – Modify protein amino acid side chains
  – Alter protein’s structure and/or aggregation state, turnover rates, activity, and protein interaction networks

• Oxidative stress is known to increase markedly in cancer, diabetes, heart disease, and neurodegenerative diseases.

• Oxidized proteins can be used as specific biomarkers of disease.

• Sample workup or storage methods can introduce artifactual oxidative modifications through exposure to dissolved oxygen, high or low pH, and/or trace metals.
Quantitative Measures of Oxidation

- **OxyELISA (Millipore)**
  - Oxygen free radicals and other reactive species introduce carbonyl groups into proteins.
  - Formation of carbonyl groups on protein amino acid side chains is one of the early markers for protein oxidation
  - Quantification of carbonyl groups following derivatization with 2,4-dinitrophenylhydrazine

- Lower limit of sensitivity is 0.2 nmol carbonyl/mg protein
- Intra-assay reproducibility CV < 9%
- Inter-assay of < 17%.
Quantitative Measures of Nitration

- **Nitrotyrosine ELISA (Northwest Life Science Specialties)**
  - Reactive nitrogen species (RNS) can be formed from nitric oxide, hydrogen peroxide, and other pro-oxidants
  - RNS can target tyrosine residues in proteins to form 3-nitrotyrosine adducts
    - A sandwich ELISA using a plate bound capture antibody (anti-nitrated KLH) to nitrotyrosine and a biotinylated secondary tracer antibody
    - Lower limit of detection is 2 nM
    - Intra-assay reproducibility CV < 8%
    - Inter-assay of < 8%.
Quantitative Measures of Aggregation

• **Size Exclusion Chromatography with UV detection**
  - Measure the fraction of protein aggregates present in the total protein content of plasma and serum
  - The SEC column functions as a molecular sieve that separates species by size
    - Column with very high $M_r$ exclusion limit ($4 \times 10^7$) to accommodate very large protein aggregates.
    - Measure “aggregate percentage value” for each sample: the ratio between the void volume peak area and total area under all peaks
Statistical Analysis

• **Quantify effects of pre-analytical variables**
  – iTRAQ data (ratio)
  – Oxidation assay (nM)
  – Nitration assay (nM)
  – SEC data (ratio)

• **For each of these four outcomes there are two main goals:**
  – Quantify the effect of procedural variables
  – Describe a normative distribution for a given sample handling procedure for quality assurance use in existing banks

• **Exploratory aim to discover a "signature" combination of peptide quantities that indicates degradation (proteolysis)**
Credentialing Plasma and Serum Biospecimen Banks

• **Plasma**
  – Colorectal cancer
  – Jim Ayers Institute for Precancer Detection and Diagnosis at Vanderbilt University

• **Serum**
  – Breast cancer
  – Early Detection Research Network sample bank at the UCSF Helen Diller Family Comprehensive Cancer Center
Clinical Translational Science Institute at UCSF

- Clinical Research Center- CRC
  - Inpatient and outpatient services
  - Nursing services
  - Bionutrition
  - Sample processing

- Consultation Services
  - Biostatistics
  - Study design and Implementation
  - Data management
  - Ethical Issues

- Training

- Working with industry and community partners
Clinical Research Center Process

• **CRC Application**
  - Study protocol and services requested
  - CHR /IRB approval

• **CRC Advisory Committee Approval**

• **Meet with CRC nursing staff**

• **Study Implementation forms**
  - MD Orders
  - Consent
  - Inclusion/Exclusion criteria
  - HIPAA

• **Blood draw**

• **Processing**
Collection Statistics

- 52 subjects completed
  - 44 samples suitable for analysis
    - Plasma collected before serum
    - Sample yield low
    - Samples not processed according to SOP
Biospecimens Database

• **mySQL-backed web-accessible electronic information system**

• **Tiered sectors**
  – Blood collection and processing, including sample tracking
  – Analytical data
  – SOPs, audit reports
  – Training records
  – Data reports

• **Password protected, limited access**

• **Servers in a controlled access high security climate controlled room located in the UCSF Library and Center for Knowledge Management**
## Biospecimens Database

### Credentialing Plasma and Serum Biospecimen Banks for Proteomics Analyses

<table>
<thead>
<tr>
<th>Donor</th>
<th>Blood Collection</th>
<th>ELISA</th>
<th>MS</th>
<th>SEC</th>
<th>SOPs</th>
<th>BioSpecimens Home</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>002</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td>(comment)</td>
<td></td>
</tr>
<tr>
<td>003</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>004</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>005</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>006</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>007</td>
<td>-- not usable</td>
<td>Serum (comment)</td>
<td>Plasma (comment)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>008</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>009</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>010</td>
<td>Serum (comment)</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>011</td>
<td>-- not usable</td>
<td>Serum (comment)</td>
<td>Plasma (comment)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>012</td>
<td>-- not usable</td>
<td>Serum (comment)</td>
<td>Plasma (comment)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>013</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>014</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>015</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>016</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>051</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>052</td>
<td>Serum</td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Links to processing data and storage location**
- **Add new data**
Serum worksheet

To be filled in at the time of blood collection and processing

<table>
<thead>
<tr>
<th>Serum Sample Label</th>
<th>Time of Collection</th>
<th>Time of Spin</th>
<th>Time of Freeze</th>
<th>Measured</th>
<th>Blood Draw Initials</th>
<th>Processor Initials</th>
<th>Auditor Initials</th>
</tr>
</thead>
<tbody>
<tr>
<td>donaldO_S_mnddyy_1R4S</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>donaldO_S_mnddyy_4C4m</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>donaldO_S_mnddyy_3F4h</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Is the serum hemolyzed? [ ] Yes [ ] No If yes, sample cannot be used.

2. Any deviations or deviations from the SOP, problems, or issues.

3. Freezer Location: [ ] 10 [ ] 100 [ ] 1 [ ] 100 [ ] 100

4. The full path & filename of the scanned image of the original worksheet:

5. This donor is usable [ ] No [ ] Yes

Save New Serum Worksheet  Cancel
## Serum Collection and Processing Data

<table>
<thead>
<tr>
<th>Serum Sample Label</th>
<th>Time of Collection</th>
<th>Time of Spin</th>
<th>Time of Freeze</th>
<th>Aliquots</th>
<th>Blood Draw Initial</th>
<th>Processor Initials</th>
<th>Auditor Initials</th>
</tr>
</thead>
<tbody>
<tr>
<td>donald0_S_manday_1T4S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>donald0_S_manday_4C4m</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>donald0_S_manday_RT4h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Is the serum hemolyzed? [ ] Yes, sample cannot be used.
2. Any variations or deviations from the SOP, problems, or issues.
3. Freezer Location: [ ] Yes, [ ] No
4. The full path & filename of the scanned image of the original worksheet:
5. This donor is usable [ ] Yes, [ ] No

### Notes
- Donor ID and date
- Time of collection, centrifugation, freezing
- Nurse, tech, auditor
- Comments, deviations from SOP
- Freezer location
- Path and filename of original worksheet
SOPs

Details, details, details...
- Procedural
- Equipment
- Standards
- Automate when possible
- Data entry
  - Documentation templates: Fields for data as well as deviations, anomalies
- Training
Outcomes

• Quantify the effect of procedural variables
  – Describe a normative distribution for a given sample handling procedure for quality assurance use in existing banks

• Exploratory aim to discover a “signature" combination of peptide quantities that indicates degradation (proteolysis)

• Generate information on inter-individual variability
  – N=50

• Generate a model of a proteomics study of cases vs. controls
  – Optimally processed samples = controls
  – Evaluate statistical approaches
Acknowledgements

• UCSF
  – Maria Hassis
  – Miles Braten
  – Matt Dahlberg
  – Evelin Szakal
  – Ewa Witkowska
  – Rich Niles
  – Susan Fisher
  – CRC Staff
    • Jennifer Barclay, Nayo Mouton-Fuentes, Clarissa Brion, Kristina Noyes, KC Medina

• SAIC Frederick
• NCI