A Protein Expression analysis of Healthy and Diseased Patient Amniotic Fluid Samples Using ProteomeLab PF 2D:

A Case Study on Sample Processing.

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4 Broad Challenges in Proteomics

1. No one technique will give all the answers.
   - Dynamic Range & Resolution Issues.
   - $10^4 - 10^5$ Genes transcribing $10^5 - 10^6$ proteins

2. The “proteome” is very dynamic.
   - Temporal & environmental influences.
   - Post-translational modifications, P-P interactions.

3. Many different protein sources.
   - Tissue, Serum/plasma, bio-fluids
   - Inter/Intra-cellular Biology & Extra-cellular Biology

4. Inclusionary vs. Exclusionary Protein Analysis
   - Whole picture vs. partial picture of expression.
   - Intact protein analysis vs. Peptide mapping.
2D Intact Protein HPLC Concept
New Gel-Free Methodologies for Proteomics

Isolation & ID of Important Protein “Targets/Markers” from Complex Mixtures

Analytical (MS) Methods

Protein Expression Arrays
Protein/Target Function Evaluation
Bioinformatics

Peptide Mapping
MALDI, LC/MS^n, CE/MS, SELDI

LC/MS - Intact Protein Analysis

EDMAN Sequencing

ProteomeLab PF2D Fractionation of Intact Proteins

ELISA’s

1D & 2D Westerns

1-D gels

Molecular Biology Methods

New Gel-Free Methodologies for Proteomics
Once you inject the sample, it’s now about the sample processing only!
Biological Samples Analyzed Using This 2D HPLC Concept

Whole Cell Lysates
Hepatocytes, Breast Cancer, Colon Cancer, Ovarian Cancer, Mouse embryonic stem cells, Yeast, E. coli, Staph Bacteria, Rat Brain Tissue, PBMC’s, Flow cytometry samples.

Protein Fluids
Secreted Proteins (conditioned media), Sera, Plasma, Amniotic Fluid, Ascites, Saliva, Urine, Various Lavages, CSF.

Misc. Protein Samples
Veterinary Vaccines, Bacterial Antigens, Bacterial spores and extracts, Plant extracts, GMO samples, Meat Product extracts, Milk/Cheese Extracts
ProteoVue pI/Hydrophobicity 2D Protein Expression Map
E. Coli O157:H7 Whole Cell Lysate

Hydrophobicity Profile for Proteins in pI range = 5.8 – 6.1

* Lane 9

pl Fractions taken at fixed pH ranges
Two Different E. coli O157 strains from Penn State
pl range 3.5 – 7; Hydrophobicity range 10 – 19 min
2D Total Protein expression map of Patient (Human) Serum

Serum samples processed the same as a cell lysate.

RT 8 – 19 min
Cellular Protein Expression vs Biofluid Protein Expression:
Sample Processing issues.

Protein Denaturation Buffer Contents
Protein Concentration/Precipitation
Selected Protein Depletion
MW cut-off filters
Contrasting Cellular analysis many biofluids have a high % of proteins with MW > 60 kDa e.g. IgA, IgG, IgM, Albumin, Transferrin, Lipoproteins.

Albumin acts as a “protein sponge” making protein expression more complicated.

Amniotic Fluid (AF) proteins are mainly serum proteins but diluted by ~ 30 fold.

Sample processing/preparation will be important to low level expression analysis.
Undepleted Serum RPHPLC Analysis
(prior to CF)

HSA sample RPHPLC Analysis
(prior to CF)
Addition of reducing agent to sample solubilization buffer breaks the albumin (transthyretin) complex!
200 uL sera in Buffer with TCEP

200 uL sera in Buffer with no TCEP

Reducing agents common additives to denature proteins!
Undepleted Human Serum RPHPLC Analysis (prior to CF)

125uL Serum undepleted

Depleted Human Serum RPHPLC Analysis (prior to CF)

125uL Serum depleted of IgG, IgA and HSA
<table>
<thead>
<tr>
<th>Undepleted Serum sample</th>
<th>HSA sample</th>
<th>Depleted Serum sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPHPLC Analysis</td>
<td>RPHPLC Analysis</td>
<td>RPHPLC Analysis</td>
</tr>
<tr>
<td>(prior to CF)</td>
<td>(prior to CF)</td>
<td>(prior to CF)</td>
</tr>
</tbody>
</table>
Both samples denatured with TCEP; They look Identical!!!
Undepleted Human Serum 2D Map
pI = 7 – 9 region

Depleted Human Serum 2D Map
pI = 7 – 9 region
Contrasting Cellular analysis many biofluids have a high % of proteins with MW > 60 kDa e.g. IgA, IgG, IgM, Albumun, Transferrin, Lipoproteins.

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ProteoVue Map 2.5 cc of EtOH precipitated AF

EtOH ppt’d resolubilized in TCEP containing Solubilization buffer
α-1 Antitrypsin Precursor

Transthyretin

Retinol Binding protein

Transferrin

Fibrinopeptide B

β-2-microglobulin

No SDS PAGE Gel Bands observed.
20 cc EtOH precipitated AF

EtOH ppt’d resolubilized in TCEP containing buffer
EtOH precipitated with TCEP gives only a partial picture of total protein expression.
Normal

Infection

12.5 cc untreated AF (RT 8-18 min)
1D gels of selected differential expressed intact proteins
Intact IGFBP-1 protein is differentially expressed in amniotic fluid.

A smaller molecular weight, anti-IGFBP-1 reactive protein, is also differentially expressed in these samples opposite to intact IGFBP-1.

The higher MW/intact isoform of IGFBP-1 is present in much greater amounts in control amniotic fluid versus samples of amniotic fluid from infected patients.

ELISA shows the total amount of IGFBP-1 (high plus low MW) does not appear to change significantly between control vs. infected.

Combined with the ELISA results the IGFBP-1 protein is cleaved upon infection resulting in the accumulation of a smaller MW form and simultaneous loss of the higher/intact isoform.

**Amniotic Fluid probed with Anti-human IGFBP-1**

- Intact IGFBP-1-Migrates slightly below 31kDa MWM
- Fragment of IGFBP-1-Migrates between 14 and 21kDa MWM

IGFBP-1 ID’d by LC/MS/MS
Filtrate from 60 kDa spin filter

RT = 7 – 18 min

12.5 cc AF
Filtrates from 60 kDa spin filter

Normal

Infection
<table>
<thead>
<tr>
<th>pl range</th>
<th>RT range</th>
<th>Normal</th>
<th>Infection</th>
<th>Protein Identification</th>
<th>Protein Identification MALDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3 – 4.6</td>
<td>12.0 – 13.0</td>
<td>Absent</td>
<td></td>
<td>P13232.13 - Interleukin-7 precursor</td>
<td>P08833.09 - Insulin-like growth factor binding protein 1 precursor (IGFBP-1) (IBP-1) (IGF-binding protein 1) (Placental protein 12) (PP12)</td>
</tr>
<tr>
<td>4.3 – 4.6</td>
<td>13.0 – 14.1</td>
<td>Absent</td>
<td></td>
<td>No confident ID</td>
<td>Q8TCG4.21 - TPMsk1 fragment</td>
</tr>
<tr>
<td>4.3 – 4.6</td>
<td>14.1 – 15.4</td>
<td>Absent</td>
<td></td>
<td>No confident ID</td>
<td>P02647.01 - Apolipoprotein A1Precursor</td>
</tr>
<tr>
<td>4.6 – 4.9</td>
<td>13.7 – 14.3</td>
<td>Absent</td>
<td></td>
<td>P31025.26 - Von Ebner's gland protein precursor (VEG protein) (Tear prealbumin) (TP) (Tear lipocalin) (Lipocalin 1)</td>
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<td>4.6 – 4.9</td>
<td>14.3 – 14.7</td>
<td>Absent</td>
<td></td>
<td>No confident ID</td>
<td>P02647.01 - Apolipoprotein A1Precursor and P02760.05 - AMBP protein precursor [Contains: Alpha-1-microglobulin (Protein HC) (Complex-forming glycoprotein heterogeneous in charge) (Alpha-1 microglycoprotein); Inter-alpha-trypsin inhibitor light chain (ITI-LC) (Bikunin) (HI-30)]</td>
</tr>
<tr>
<td>4.6 – 4.9</td>
<td>16.4 – 17.2</td>
<td>Absent</td>
<td></td>
<td>No confident ID</td>
<td>P02647.01 - Apolipoprotein A1Precursor and P01009 - Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor) (Alpha-1-antiproteinase)</td>
</tr>
<tr>
<td>4.9 – 5.2</td>
<td>12.8 – 13.3</td>
<td>Absent</td>
<td></td>
<td>P31025.26 - Von Ebner's gland protein precursor (VEG protein) (Tear prealbumin) (TP) (Tear lipocalin) (Lipocalin 1)</td>
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</tr>
<tr>
<td>5.2 – 5.5</td>
<td>13.9 – 14.3</td>
<td>Absent</td>
<td></td>
<td>No confident ID</td>
<td>P51812 - Ribosomal protein S6 kinase alpha 3</td>
</tr>
<tr>
<td>5.2 – 5.5</td>
<td>15.8 – 16.4</td>
<td>Differential Expression of 3 prominent bands (previously found α-1 antitrypsin in this area)</td>
<td></td>
<td>P02647.01 - Apolipoprotein A1 Precursor_only 1 peptide ID</td>
<td>P02647.01 - Apolipoprotein A1Precursor</td>
</tr>
</tbody>
</table>

**EPROGEN INC**
Understanding the effects of sample processing is key to interpreting protein expression results.

1. Reducing Agents alters/influences protein expression especially w.r.t. albumin.

2. Protein Precipitation effective for dilute biofluids.

3. Selected Protein Depletion: Effective but do reducing agents have the same effect as albumin depletion?

4. MW cut-off filters are a great way to isolate and ID low expressed proteins in high albumin containing biofluids.
"You've got one protein missing..."
"No, you've one extra protein!"