Techniques in Gel Electrophoresis and Western Blotting

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History of Gel Electrophoresis

- Raymond and Winstraub (1959) introduced acrylamide gels
- Shapiro et al. (1967) and Beber and Osborn (1969) reported improved separation with SDS
- Laemmli (1970)
  - Stacking gel – discontinuous gel
  - Discovered unknown proteins in bacteriophage T4
- Method used across countless disciplines
  - Meat Science
1-D Gel Electrophoresis

- SDS-PAGE
- Charged molecules move in response to an electric field
  - Net charge
  - Resistance
SDS-PAGE

- Sodium dodecyl (lauryl) sulfate (SDS)
  - Solubilizes proteins
  - Primary structure
  - Imparts a net negative charge approximately proportional to the mass of each protein
  - Negatively charged proteins will migrate toward the positive anode
    - Mobility is a function of molecular weight

\[
\text{H}_3\text{C}-(\text{CH}_2)_{11}-\text{O-S-O}^\cdot\text{Na}^+ \]

SDS-PAGE

- Polyacrylamide gel electrophoresis (PAGE)
  - Chemically inert matrix
  - Variations in pore size alters:
    - The extent of migration
    - Extent of protein separation
    - Molecular weight range
Altering pore size

- Acrylamide percentage
  - 3% to 20%
  - Shifts the molecular weight range of proteins resolved

- Acrylamide:bisacrylamide
  - 37.5:1 to 200:1
  - Slight modification in resolving power
SDS-PAGE

- Other alterations
  - Changing pH of resolving gel (Makowski and Ramsby, 1993)
    - 8.8 to 9.2
    - Improved resolution of low molecular weight (<20 kDa) proteins
  - Voltage
    - Lower voltage over a longer period of time improves resolution
Limitations of SDS-PAGE

- Migration of high molecular weight proteins is limited
  - 3-5% acrylamide gels
  - 3-12% gradient gels
    - Challenging to pour
    - Physically difficult to handle
    - Undergo physical distortion or tearing during staining
    - Transfer of large molecular weight proteins for western blotting is challenging
Limitations of SDS-PAGE

- Addition of agarose to 2% acrylyamide gels (Huebsch et al., 2005; Tatsumi and Hattori, 1995)
  - Improves separation of $T_1$ and $T_2$
  - Limited ability to separate titin isoforms

- Vertical Agarose gel electrophoresis (VAGE)
  - Developed by Warren et al. (2003)
  - Improved separation of:
    - Cardiac titin isoforms N2B, N2BA
    - Intact titin ($T_1$) and the breakdown product of titin ($T_2$)
SDS-VAGE Overview

- Relies on the same basic principles as PAGE gels
  - Sample preparation
    - Gentle homogenization to minimize damage to large proteins
    - Sample buffer: 8 M urea, 2 M thiourea, 3% w/v SDS, 75 mM DTT, 0.03% bromophenol blue, and 0.05 M Tris-HCl, pH 6.8
    - Denatured at 60°C for 10 min
  - Sandwich assembly
    - 1.5 mm T-spacers
    - ~1cm high 12% acrylamide plug
    - Warmed at ~60°C
SDS-VAGE Overview

- **Resolving gel**
  - 1% w/v agarose (Sea Kem gold agarose), 30% v/v glycerol, 50 mM Tris base, 0.384 M glycine, and 0.5% SDS
  - Solution is heated and poured into the warmed sandwich assembly
  - 1.5mm combs are immediately placed in the agarose

- **Electrophoresis**
  - Lower chamber buffer (50mM Tris base, 0.384 M glycine, and 0.1% SDS)
  - Samples loaded through the upper buffer (lower chamber buffer with 10 mM β-mercaptoethanol)
  - Run at 15 mA constant current for ~5 h at 4°C
SDS-VAGE Advantages

- Improved mobility of titin
- Resolution of titin isoforms
- Improved separation of T1 and T2 fragment
- Improved gel stability
- Useful for characterizing other large molecular weight proteins

Warren et al., 2003
SDS-VAGE Advantages

- Nearly 100% transfer efficiency

Warren et al., 2003
SDS-VAGE Limitations

- Prevention of air bubbles in resolving gel
- Premature solidification of agarose
- Removal of comb from solidified agarose gel
- Limited storage capability
  - 1-2 d at 4°C
Two-Dimensional (2-D) Gels

- Very effective tool for separating complex mixtures
  - Separates on BOTH electric charge and size
- First dimension
  - Isoelectric focusing gel
    - Separates proteins on charge
- Second dimension
  - Separates proteins on size
2-D gels

pH 3  pH 10  MW

75 kDa
50 kDa
25 kDa
# Acrylamide gel Visualization

<table>
<thead>
<tr>
<th>Stain</th>
<th>Sensitivity in ng</th>
<th>Staining Time</th>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie Blue</td>
<td>36-47</td>
<td>30 min-6 hr</td>
<td>simple, consistent</td>
</tr>
<tr>
<td>Silver Stain</td>
<td>0.6-1.2</td>
<td>60-90 min</td>
<td>sensitive</td>
</tr>
<tr>
<td>Copper</td>
<td>6-12</td>
<td>10 min</td>
<td>non-fixative</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Negative Stain</td>
</tr>
<tr>
<td>Zinc</td>
<td>6-12</td>
<td>10 min</td>
<td>non-fixative, higher contrast</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Negative Stain</td>
</tr>
</tbody>
</table>
Staining

- Coomassie Blue R-250
Staining

- Silver Staining
Staining

- Negative Stains

Zinc Stain
**Fluorescent Stains**

Two stains shown below do not stain DNA or RNA
Can be a problem with other sensitive stains like Silver Stain

<table>
<thead>
<tr>
<th>Sypro Orange</th>
<th>Sypro Ruby</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity greater than Coomassie</td>
<td>Sensitivity equivalent to silver stain (1–10 ng)</td>
</tr>
<tr>
<td>2-10 ng/band</td>
<td>Proteins are fixed in the gel</td>
</tr>
<tr>
<td>Rapid - 30 minutes</td>
<td>*Detects proteins that are not stained well by other stains (glycoproteins, lipoproteins, metalloproteins)</td>
</tr>
<tr>
<td>Reversible - can use gel for immuno blotting</td>
<td></td>
</tr>
</tbody>
</table>
2-D DIGE
(Two-Dimensional Fluorescence Difference Gel Electrophoresis)
2-D electrophoresis

Sample A
Label with Cy3 dye
Mix

Sample B
Label with Cy5 dye

Excitation wavelength for Cy3
Overlaid Image

Excitation wavelength for Cy5
Immunoblotting
(Western Blotting)

- Immunoblotting
- Identification of specific proteins fractionated on acrylamide gels
Immunoblotting (Western Blotting)

- Powerful extension of SDS-PAGE
  - Separation of proteins with SDS-PAGE
  - Transfer of proteins to suitable membrane
  - Application of antibody specific for a protein (1° Ab)
  - Application of antibody specific for 1° Ab
    - 2°Ab - tagged with a reporter molecule
  - Reporter molecule activated
SDS-PAGE Gel

Nitrocellulose or PVDF membrane
SDS-PAGE Gel

Nitrocellulose or PVDF membrane
Assembly of transfer unit
Immunoblotting

15% gel

Troponin-T
Optimizing Immunoblotting

- **Signal intensity**
  - protein load on gel
  - Transfer of protein from gel
  - membrane
  - transfer buffer composition
  - transfer time/temperature
  - antibody
  - 2° tag

- **Background**
  - blocking
  - choice of antibody
  - antibody concentration
## Optimizing Immunoblotting

### Blocking

<table>
<thead>
<tr>
<th>Blocker</th>
<th>Composition</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFDM/Tween</td>
<td>5% NFDM and 0.1-0.5% Tween</td>
<td>cheap, clean background</td>
<td>limited storage interferes with biotinylated samples</td>
</tr>
<tr>
<td>BSA</td>
<td>3% (IgG free) and 0.1-0.5% Tween</td>
<td>good signal</td>
<td>relatively expensive</td>
</tr>
<tr>
<td>Gelatin</td>
<td>3% and 0.1-0.5% Tween</td>
<td>inexpensive little interference with biotinylated samples</td>
<td>may gelatinize at room temperatures</td>
</tr>
</tbody>
</table>
Detection Methods

Color Development Systems

4-chloro-1-napthol (4CN)
Oxidation of luminol by HRP/H$_2$O$_2$ in the presence of phenolic chemical enhancers

Light emission 428-450 nm

Signal be captured with blue-light sensitive autoradiography film or phosphorimaging devices
Detection Methods

4-chloro-1-naphthol

chemiluminescence
ECL-Plex
2-color western blotting
So…. What is the take-home message?

- All of these technologies have the capacity to provide exciting new information for meat/muscle biology research.
- The key is to ask the correct question and then match the appropriate technology.
  - MUST understand the limitations of each method and know how to modify them if needed.

"A problem well-stated is a problem half solved."
--Charles Kettering, 1876–1958, farmer, teacher, mechanic, engineer, scientist, inventor and social philosopher.
Questions????