Lesson 9: Protein electrophoreses

➢ Native gel
  ➢ (Band shift/retardation assay)

➢ Denaturing gel (SDS-PAGE)

➢ Isoelectrofocusing

➢ 2D Gel electrophoresis
  ➢ (Native-blue PAGE)
  ➢ (Western blot)
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Example: electrophoresis based on molecular weight
Electrophoresis

- Technique mainly devoted to analytical aims, with a few preparative applications

- Macromolecules are separated on a gel matrix upon the application of an electric field, according to their shape, size and charge

- Shape, size and charge of proteins influence their electrophoretic mobility ($\mu$)
Velocity of migration and mobility of a particle

\[ v = \mu \cdot E \]

\[ \mu = \frac{q}{f} \]

\[ f = 6\pi \eta R_h \]

E, electric field
q, charge
v, velocity of particle
h, viscosity coefficient of the medium
R_h, hydrodynamic radius (Stokes’ radius)

\[ \mu = \frac{q}{6\pi \eta R_h} \]

Mobility depends:
  - Directly on the particle charge, q (in coulomb);
  - Directly on electric field, E (in volt/cm);
  - Inversely on friction coefficient, f

Particles must be charged!!
Where are protein charges coming from?

Polar group ionization

Native gel

Sodium Dodecyl Sulfate (SDS)

Denaturing gel
Native PAGE

- Native PAGE gel do not contain any denaturing agents
- Electrophoretic mobility depends on shape, dimension and net charge of the native protein
- The electrostatic charge depends on pI and pH
- Shape and size ($R_H$) depends on MW, tertiary and quaternary structure
- The sense of run can change
Native PAGE

Negatively charged proteins  Positively charged proteins
Plasma Protein Distribution

7.6 g protein / 100 mL serum

- Albumin (60%)
- Globulin (35%)
- Fibrinogen (4%)
- Other plasma proteins (1%)

Alpha 1 globulin
Alpha 2 "
Beta 1 "
Beta 2 "
Gamma "

RED BLOOD CELLS
PLATELETS
PLASMA
WHITE BLOOD CELLS
Serum Protein electrophoresis on agarose gel

Principle:
• At pH 8.6 serum proteins have negative charges and they move toward the anode at rate dependent on their net charge.
• Once separated, proteins are fixed, stained and subjected to densitometric analysis (comparison with standards)

Device:
Serum protein electrophoresis on agarose gel is a type of horizontal gel electrophoresis

At the end of run: 1. Fixing with 80% picric acid and acetic acid 20% (v/v). 2. Staining with Blue Coomassie

http://www.mun.ca/biology/desmid/brian/BIOL2250/Week_Three/electro4.jpg
GEL ELECTROPHORESIS

Sebia agarose gel electrophoresis:
A well-established and robust protein separation technology
Serum Protein electrophoresis on agarose gel

Serum proteins are separated into 6 groups:

- Albumin
- α1 - globulins
- α2 - globulins
- β1 - globulins
- β2 - globulins
- γ - globulins

Figure modified from http://www.sebia.com/en-US/produits/hydragel-b1-b2
Clinical biochemistry assays

- Gels with 15 or 30 wells (serum samples) are used in clinical laboratories.
- Electrophoresis is also applied to separate isoenzymes, nucleic acids and immunoglobulins

Densitometry of separated protein fractions

Densitometer is used for scanning of protein patterns giving a quantitative information about protein fractions

Signal intensity (number of pixels)

albumin

α₁  α₂  β₁,₂  γ
Where are protein charges coming from?

**Native gel**

Polar group ionization

**Denaturing gel**

Sodium Dodecyl Sulfate (SDS)

Catodic migration

Anodic migration

Net change of protein

Isoelectric point
SDS-PAGE
(sodium dodecyl sulfate polyacrylamide gel electrophoresis)

➢ Principles
➢ Staining
➢ Calibration
➢ Application to determine MW and purity degree
SDS: sodium dodecyl sulfate

- Ionic detergent used in electrophoresis to confer negative charge to proteins
- Binding ratio: one molecule of SDS every 2 residues (1 SDS / 2aa), mainly reflecting the average frequency of apolar residues
- Binding to SDS makes homogeneous charge:mass ratio $\rightarrow$ proteins separated according to their mass

SDS charge stands on residues charges $\rightarrow$ denaturing effects
Reducing agents

- SDS is ineffective on covalent bonds, included S-S (between SH groups of Cys residues)
- Reducing agents reduce S-S bonds, thus completing the loss of secondary and tertiary structure

β-mercaptopethanol

DTT - dithiothreitol

tris(2-carboxyethyl)phosphine
In SDS-PAGE separation is based on molecular weight

- Similar shape (extended/denatured)
- Similar charge (ratio charge/MW = charge density)
- Different molecular weight (MW)
Estimating molecular size by determining electrophoretic mobility
Matrices for gel electrophoresis

- Gel matrix is a net and can be crossed by particles
- Particles are slowed down according to mesh size
- Principle: molecular sieve (similar to gel filtration, with opposite effect)

Examples

- small-meshed matrix (polyacrilamide gel) for proteins and small nucleic acids
- large-meshed matrix (agarose gel) for high-weight nucleic acids
Size of matrix mesh depends on acrylamide concentration

Gel matrix
Size of matrix mesh depends on acrylamide concentration

Acrylamide concentration and range of protein fractionation

<table>
<thead>
<tr>
<th>Acryl. Concentr. (%)</th>
<th>MW range (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>60-350</td>
</tr>
<tr>
<td>10</td>
<td>15-200</td>
</tr>
<tr>
<td>15</td>
<td>10-100</td>
</tr>
</tbody>
</table>
Acrylamide polymerization

Acrylamide polymerizes in the presence of ammonium persulphate (AP) and tetramethylethylendiamine (TEMED). TEMED catalyzes the decomposition of AP ion and produces free radicals.

\[
\begin{align*}
S_{2}O_{8}^{2-} & \rightarrow 2SO_{4}^{2-} \\
\text{radical AP radical}
\end{align*}
\]
Polyacrylamide gels are obtained by copolymerization of acrylamide and bis-acrylamide ("Bis" - N,N'-methylene-bisacrylamide). The polymerization reaction is a vinyl addition initiated by a system of free radical generation (Chrambach 1985). The polymerization is initiated by ammonium persulfate and TEMED (tetramethylthylene diamine): TEMED accelerates the formation of free radicals from ammonium persulfate and this in turn catalyzes the polymerization. The free radicals of ammonium persulfate transform the monomer of acrylamide into free radicals, which react with monomers to give rise to a chain polymerization (Shi and Jackowski 1998). The polymer chains are intercrosslinked by the "bis". The result is the formation of a gel whose porosity depends on the polymerization conditions and the monomer concentration when ammonium persulfate is dissolved in water forms free radicals.
Polymerization is carried out in the presence of small amount of bis-acrylamide (cross-linker)
Use of acrylamide gradient concentration

12% kDa
10-20% kDa

- 175,0
- 83,0
- 62,0
- 47,5
- 32,5
- 25,0
- 212
- 2.3
Commercially available precast gels

<table>
<thead>
<tr>
<th>Gel %</th>
<th>Size range (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-15</td>
<td>20-200</td>
</tr>
<tr>
<td>5-20</td>
<td>10-200</td>
</tr>
<tr>
<td>8-15</td>
<td>10-100</td>
</tr>
<tr>
<td>8-20</td>
<td>8-150</td>
</tr>
<tr>
<td>10-20</td>
<td>6-150</td>
</tr>
</tbody>
</table>
How to run SDS-PAGEs
Discontinuous gel make more efficient the separation.
Stacking gel: proteins stacked in a narrow band before the separation in the running gel.
At the beginning of electrophoresis

- Cl⁻ (green) fastest
- Glycine (orange) slowest in the stacking gel (pH~ 5.97 = pI of glycine)
- Proteins have intermediate mobility (in between Cl⁻ and glycine)
Charged forms of glycine

\[ \text{pl} = 5.97 \text{ (net charge 0)} \]
At \( \text{pH} > 5.97 \) net charge < 0
At \( \text{pH} < 5.97 \) net charge > 0
At the start

- Cl⁻ (green) in the stacking and resolving gels
- Glycinate (orange) in the tanks

In the stacking gel

Proteins stacked into a thin layer between Cl⁻ and glycinate

In the resolving gel

Separation according to MW
Protein staining with Coomassie blue

Electrostatic, hydrophobic, van der Waals interactions between Coomassie blue and proteins
Silver staining of proteins

- 100 fold more sensitive than Coomassie blue
- Reduced $\text{Ag}^+$ to metallic Ag is deposited on proteins ($\text{Ag}^*$ is reduced by formaldehyde)
Examples

Native gel  SDS-PAGE

homo-oligomers  (with heterogeneous stoichiometry)

UDP-glucose pyrophosphorylase (UGPase)

Native gel  SDS-PAGE

hetero-dimer  (with unique stoichiometry)

leucyl-tRNA synthetase (LeuRS)
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Isoelectrofocusing (IEF)

- Isoelectrofocusing separates proteins according to their pI, in a gel containing a pH gradient.
- Under an electric field, migration occurs until a protein encounters a pH equal to its own pI. Then, the net charge is null and the protein stops.
Mechanism of focalization

(A) Low pH (+) → High pH (-)

(B) Low pH (+) → High pH (-)

Figure 3.11
Biochemistry, Seventh Edition
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http://oregonstate.edu/instruct/bb450/450material/stryer7/3/figure_03_11.jpg
Example of run (pH range 5-10)

- $pH_{\text{max}} = 10$
- $pH_{\text{min}} = 5$

Protein charges at the basic end:
- $p_I = 11$
- $p_I = 7$
- $p_I = 5$
- $p_I = 4$

Sense of migration:
- If $p_I > pH_{\text{max}}$, proteins do not enter the gel.
- If $p_I < pH_{\text{max}}$, proteins go out the gel.
Which conditions to run an IEF?

NATIVE | DENATURING
Composition of IEF sample buffer

- Urea (denatures without shielding the natural charges)
- Non ionic detergent (CHAPS, NP-40, Triton X-100)
- Reducing agent (DTT, beta-mercaptoethanol,...)

Denaturing conditions are applied to separate subunits in quaternary structure
Immobilization of pH gradient

Acrylamido buffers (immobilized acidic or basic groups)

\[
CH_2=CH-CO-NH-R \quad R \text{ contains a carboxylic or a tertiary amino group}
\]

Preparation of pH gradient gel

Acrylamide with acidic buffering property

Acrylamide with basic buffering property

Gel with immobilized gradient (IG) of pH

0.5 mm gel on film support

pH gradient from 3 to 10
Devices to run IEF-IG
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Samples (50-100 μg) solubilized in urea 9 M, CHAPS 0.8%, DTT 15 mM
Do not use SDS
Use of IEF-IG strips. II

3. Strips are removed after electrophoresis

4. Soaking in denaturing buffer
   - Tris-HCl 50 mM pH 8.8, urea 6M, glycerol 30% (w/v), SDS 2% (w/v). Incubation for 15 min

Gel w/o wells
Strip sealed with agarose

5. Strip inserted in SDS-PAGE
   - Marker MW
Bidimensional electrophoresis (2D gels)

First dimension on strips
Second dimension on slab gel
2D gels

Proteomics: science that studies in the whole all the proteins from an organism, a tissue, a cell line
Proteins under/over-expressed can be extracted from a gel and identified by mass spectrometry.
Detection of phosphorylation isoforms

3, 2, 1, 0 phosphate groups

steroidogenic acute regulatory protein (StAR)

Pros & cons of 2D-electrophoresis

**Pros:**
- Able to separate proteins with pl ca. 0.0025 pH units
- Relative amount of protein quantified by intensity
- Provides clear patterns of protein expression
- Comparison can identify protein expression differences
- Suitable to identify novel proteins

**Cons:**
- Unable to identify all proteins based on pl/size alone
- Unsuitable to analyse extreme acidic/basic proteins
- Misses some large proteins and membrane proteins
- Limited reproducibility of 2D gels
- Relatively slow, expensive process
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Coomassie (G250) colors

- **pH ~ 0**
  - Net charge = +1
  - λ max = 470 nm

- **pH ~ 1**
  - Net charge = 0
  - λ max = 620 nm

- **pH = 7**
  - Net charge = -1
  - λ max = 595 nm

Coomassie dyes are used at pH 7 to bind proteins in ‘blue-native PAGE’
Native blue (NB) PAGE

Protein samples (native conditions) + Coomassie G-250 → Protein complexes → Electrophoresis

- Coomassie dyes at pH 7 give negative charge to proteins
- The mobility of complexes depends on this charge and on molecular weight
- The complexes remain native

Interaction with Coomassie can destabilize weak complexes

2D con 1° dimensione non denaturante per analizzare proteine con struttura quaternaria o complessi proteici
Mitochondrial protein complexes were first separated by BN-PAGE. Then, protein bands (corresponding to protein complexes) were cut out from the gel, and electroeluted.

Finally, the subunits of each complex were resolved by IEF/SDS-PAGE. The isoelectric points and molecular masses of standard proteins are given above and to the left of the gel.

A fruitful procedure for the generation and analysis of subproteomes (subsets of proteins of the eukaryotic cell)

Werhahn and Braun, Electrophoresis (2002) 23, 640–646
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Gel retardation (band shift) assay

Gel non denaturante per studiare interazioni DNA proteina

Stessa quantità di DNA marcato con sequenza riconosciuta da una proteina

Native PAGE

Rilevazione DNA marcato

DNA legato

DNA libero
Gel retardation (band shift) assay

Gel non denaturante per studiare interazioni DNA proteina

DNA con sequenza riconosciuta da antigene nucleare del virus Epstein-Barr (EBNA)
Tryptophan repressor protein and DNA

Elettroforesi e calore

La maggior parte dell’energia trasferita durante l’elettroforesi viene dissipata sotto forma di calore.
Il riscaldamento del mezzo elettroforetico ha i seguenti effetti:

• Un aumento della diffusione del campione e degli ioni che porta ad allargare i campioni separati (Perdita di risoluzione)
• La formazione di correnti di convezione con conseguente miscelazione di campioni separati
• Denaturazione termica delle proteine (e quindi la perdita di attività enzimatica)
• Una diminuzione della viscosità del tampone e quindi una riduzione della resistenza del mezzo.
• Deformazione del supporto elettroforetico
Durante l’elettroforesi l’aumento di temperatura provocato dalla potenza dissipata diminuisce la resistenza elettrica del mezzo poiché diminuisce la viscosità e l’evaporazione concentra gli ioni. Questo rallenta la migrazione del campione.
Per evitare il surriscaldamento della cella, che si rischierebbe utilizzando una tensione costante, si imposta l’alimentatore a corrente costante.
Durante l’elettroforesi si ha quindi una caduta del voltaggio con conseguente riduzione della velocità di migrazione.
Mantenendo costante la corrente anche la potenza dissipata nel corso della separazione si riduce.