Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2018

Lecture 24:

Analysis of Gel Images and Some Other Electrophoretic Techniques

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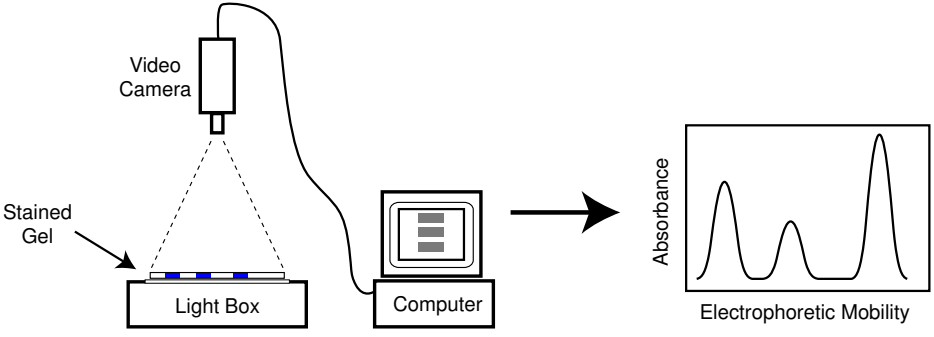
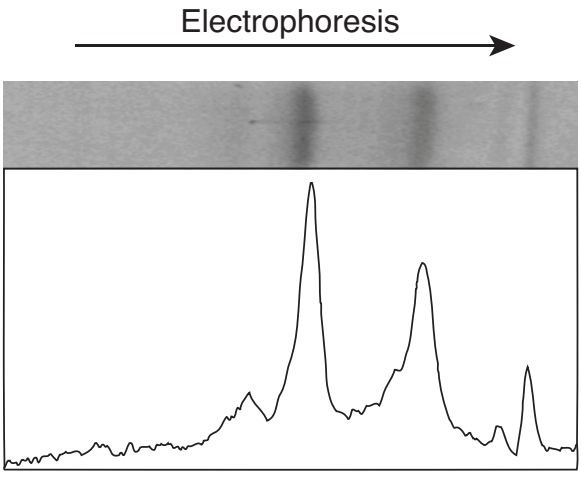
Video Densitometry of Gels



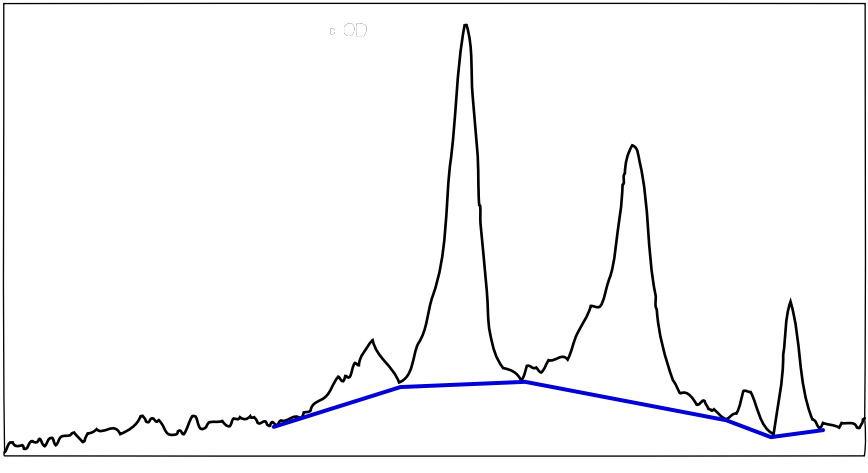
- Lightbox and camera function as a position-specific spectrophotometer.

Scans of SDS Gel Lanes



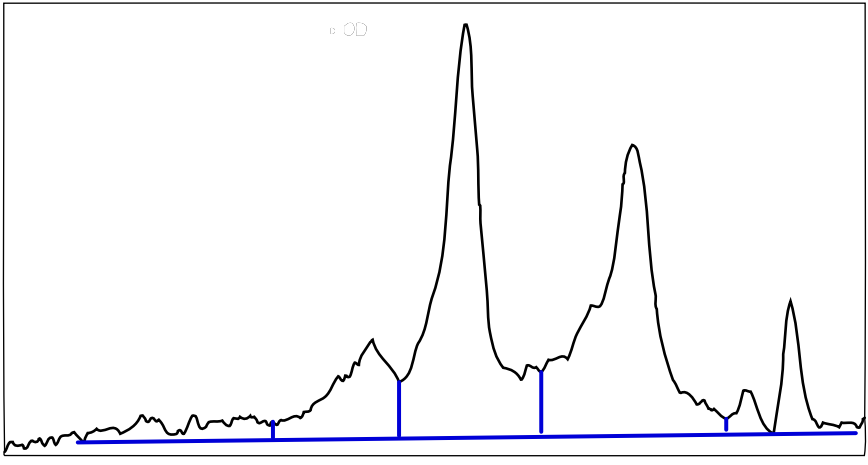
- Integrals of peaks are proportional to protein concentrations.
- Have to define limits of individual peaks and baseline.

One Way to Draw Baselines



- Implies that there is a broad distribution of stain below the peaks that is unrelated to protein concentration.

Another (Correct) Way to Draw Baselines



Kinetic Analysis of the RNAse A Digestion Experiment

- Michaelis-Menten Equation: $V = \dfrac{[\mathrm{E}]_\mathrm{T} k_\mathrm{cat} [\mathrm{S}]}{K_\mathrm{m} + [\mathrm{S}]}$

- If $[\mathrm{S}] \ll K_\mathrm{m}$:
  (We will assume this as a simplification, without much justification.)

$$V = \frac{[\mathrm{E}]_\mathrm{T} k_\mathrm{cat} [\mathrm{S}]}{K_\mathrm{m}}$$

- A first-order rate expression: $\dfrac{d[\mathrm{S}]}{dt} = -V = -k_\mathrm{app}[\mathrm{S}]$

  where:

$$k_\mathrm{app} = \frac{[\mathrm{E}]_\mathrm{T} k_\mathrm{cat}}{K_\mathrm{m}}$$

The Time Course for Digestion

- First-order rate expression:

$$\frac{d[\mathrm{S}]}{dt} = -k_{\mathrm{app}}[\mathrm{S}]$$

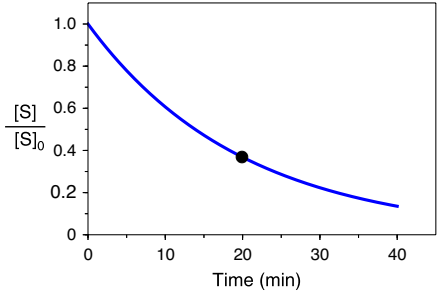
- After integration:

$$\frac{[\mathrm{S}]}{[\mathrm{S}]_0} = e^{-k_{\mathrm{app}} \cdot t}$$

$[\mathrm{S}]_0 =$ initial substrate concentration

- Take logarithms and solve for k_{app}:

$$k_{\mathrm{app}} = -\frac{\ln\left([\mathrm{S}]/[\mathrm{S}]_0\right)}{t}$$



Clicker Question #1

If 70% of the protein is cleaved in 20 min,
what is the apparent first-order rate constant, k_{app}?

1. $3 \times 10^{-4}\ s^{-1}$
2. $1 \times 10^{-3}\ s^{-1}$
3. $2 \times 10^{-2}\ s^{-1}$
4. $6 \times 10^{-2}\ s^{-1}$

$$k_{app} = -\frac{\ln\left([S]/[S]_0\right)}{t} = -\frac{\ln\left(1 - 0.7\right)}{20\ \text{min}} = 0.06\ \text{min}^{-1}$$

$$= 0.06\ \text{min}^{-1} \times \frac{1\ \text{min}}{60\ \text{s}} = 0.001\ \text{s}^{-1}$$

Estimating k_{cat}/K_m from a Single Time Point

- From integrated rate expression:

$$\ln\left([S]/[S]_0\right) = -k_{app} \cdot t$$

$$k_{app} = -\frac{\ln\left([S]/[S]_0\right)}{t}$$

- Calculate k_{cat}/K_m from k_{app} and $[E]_T$:

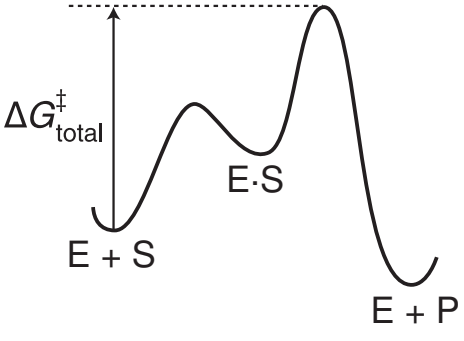
$$k_{app} = \frac{[E]_T k_{cat}}{K_m}$$

$$k_{cat}/K_m = k_{app}/[E]_T$$

The Significance of k_{cat}/K_m

- If $K_d \approx K_m$:

$$\Delta G^{\ddagger}_{total} = C - RT \ln\left(\frac{k_{cat}}{K_m}\right)$$



- Catalytic efficiency is favored by a large value of k_{cat} and a small value of K_m.
- How does k_{cat}/K_m compare for a protein and a synthetic substrate?

Methods for Detecting Proteins in Gels

- To detect all proteins:
  - Staining with coomassie blue:
    Dye binds to proteins and excess is washed away
    Sensitivity: ≈ 0.1–$1\ \mu$g/band
  - "Silver staining":
    Based on reduction of silver ions to metallic silver in vicinity of protein.
    Much more sensitive than coomassie blue; as little as 10 ng/band
    Messy, finicky and sensitive to artifacts.
  - Staining with fluorescent dyes
    Dye fluorescence is enhanced when bound to proteins
    Much more sensitive than coomassie blue
  - Other dyes and metal ions.
- To detect specific protein classes:
  - Specific dyes for phosphoproteins
  - Specific dyes for glycoproteins

Steinberg, T. H. (2009). Protein gel staining methods: An introduction and overview. *Methods Enzymol.*, 463, 541–563. http://dx.doi.org/10.1016/S0076-6879(09)63031-7

Detection of Gel-fractionated Proteins with Antibodies: Western Blotting

- Allows selective detection of specific proteins: Requires specific antibodies.
- After electrophoresis, proteins are electrophoresed out of the gel and transferred (“blotted”) onto a membrane (usually nitrocellulose or polyvinylidene difluoride)

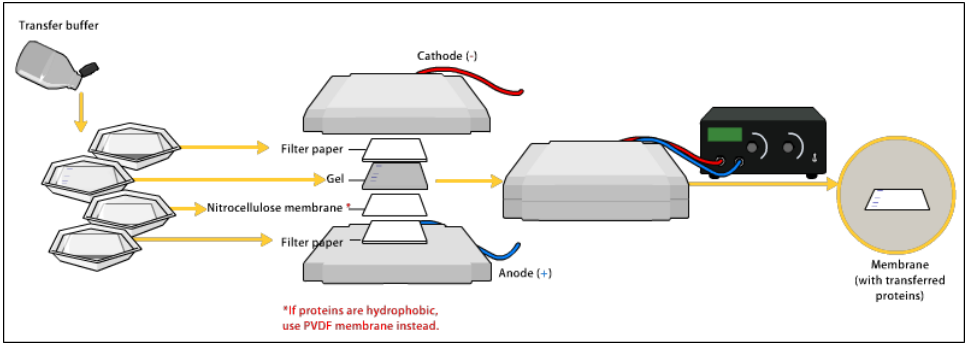
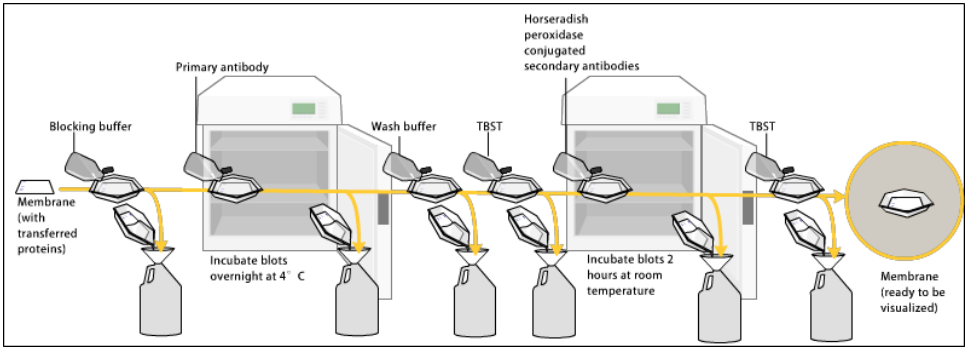


Illustration from `https://en.wikipedia.org/wiki/Western_blot`

Detection of Gel-fractionated Proteins with Antibodies: Western Blotting

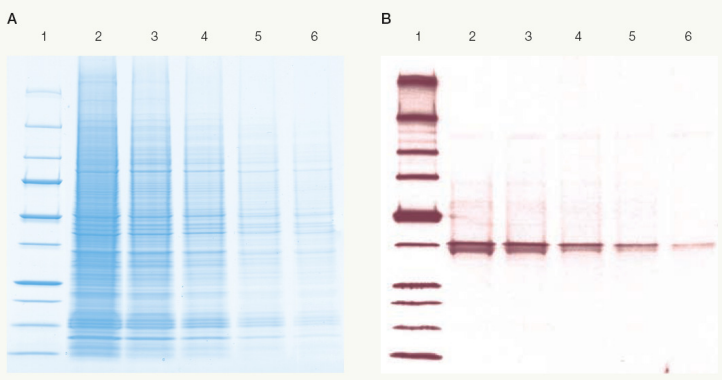
- Multistep treatment of membrane with bound proteins.



  - Generic protein solution to block all protein binding sites.
  - “Primary” antibody specific for protein of interest.
  - “Secondary” antibody, binds to primary antibody and is labeled for detection. Radioactive label or enzyme that generates colored precipitate or chemiluminescence.

Illustration from `https://en.wikipedia.org/wiki/Western_blot`

Western Blot Example



Coomassie blue Western blot

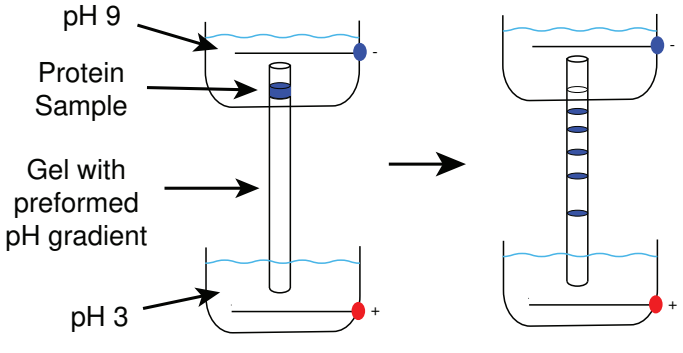
- Sample: HeLa cell lysate.
- Antibody: Specific for human CDK7 protein. (cyclin-dependent kinase)

Illustration from Bio-Rad Bulletin 2032 Rev. E,
www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_2032.pdf

A Bit More About Western Blots

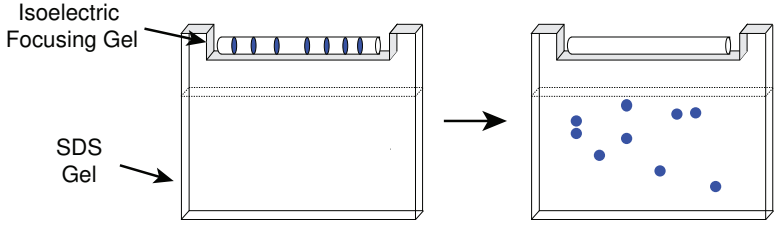
- Clinical applications: Used to detect antibodies in patients indicative of infection.
  - HIV
  - Lime disease
  - Hepatits
  - Other viral and bacterial infections
- Where does the name come from?
  - 1975: DNA blotted from gels and detected by hybridization to radiolabeled DNA. Invented by Edwin Southern.
    https://doi.org/10.1016/S0022-2836(75)80083-0
  - 1977: RNA blotted from gels and detected by hybridization to radiolabeled DNA. Invented by James C. Alwine, David J. Kemp, and George R. Stark and nicknamed by others “northern blotting”.
    https://dx.doi.org/10.1073%2Fpnas.74.12.5350
  - 1981: Proteins plotted from gels and detected by antibody binding. Invented by W.Neal Burnette, who called it “western blotting”.
    https://doi.org/10.1016/0003-2697(81)90281-5

Another Electrophoresis Method: Isoelectric Focusing (IEF



- pH gradient is formed by mixtures of buffering compounds incorporated in the gel.
- Proteins migrate to point where they have no net charge, and then stop.
- Proteins are separated by isoelectric point.
- Usually performed in the absence of detergent, but often with urea present to unfold proteins.

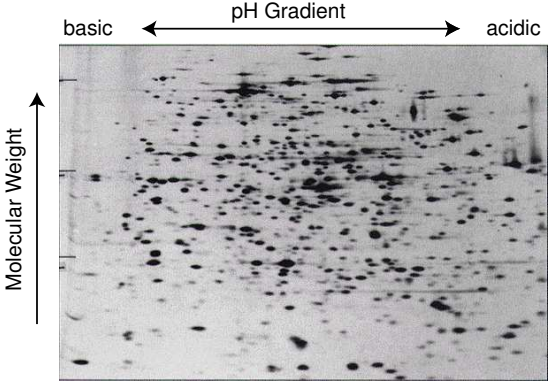
Two-dimensional Gel Electrophoresis



- Isoelectric point and chain molecular weight are largely independent properties.
- Greatly increases ability to resolve proteins in complex mixtures.
- Simultaneously provides information about molecular weight and isoelectric point.
- Other variations are possible (*e.g.,* native gel/SDS gel)

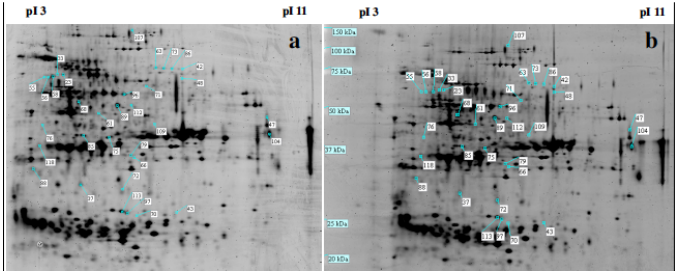
O'Farrell, P. H. (1975). High resolution two dimensional electrophoresis of proteins. *J. Biol. Chem..*, 250, 4007–4021. http://www.jbc.org/content/250/10/4007.abstract

A 2-dimensional Gel of Bacterial Proteins



2-dimensional Gel Electrophoresis in Proteomics

- Proteomics: A (sort of) new discipline focused on analysis of protein compositions of cell, tissues and organisms under different physiological, developmental or environmental conditions. One of many new “omics”!
- An example using 2-d gel electrophoresis:



Comparison of cultured tissue (callus) of vanilla orchid under different conditions.

- Spots in gel can be excised and chemically analyzed to identify proteins: Protease digestion, mass spectrometry, sequencing

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Palama, T. L., Menard, P., Fock, I., Choi, Y. H., Bourdon, E., Govinden-Soulange, J., Bahut, M., Payet, B., Verpoorte, R. & Kodja, H. (2010). *BMC Plant Biology*, 10, 82.