A Basic Primer to Protein Electrophoresis

Roger Biringer

1D- IEF

- Isoelectric Focussing (IEF)
  - Ampholyte-Based Gels:
  - Polyacrylamide or agarose gels are cast in the presence of ampholytes, a mixture of polymeric buffers with a continum of pIs.
  - Under the influence of an applied electric field, hydroxide ion and hydronium ion migrate from the cathode and anode respectively to rapidly form an unbuffered pH gradient across the gel slab. Slower migrating ampholytes move through the gel to the point where the pH = pI and serve to buffer the pH gradient.
  - The pH gradient drifts to cathode with time, placing a restriction on the allowed focussing time.

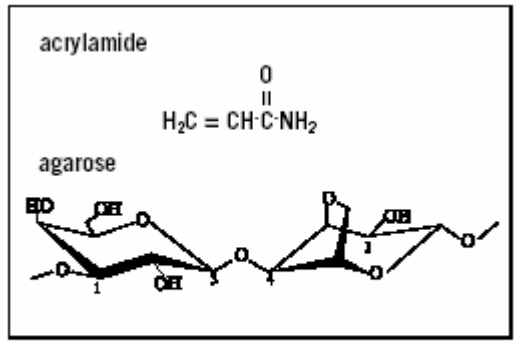


Fig 1.2. Chemical structure of acrylamide and agarose.

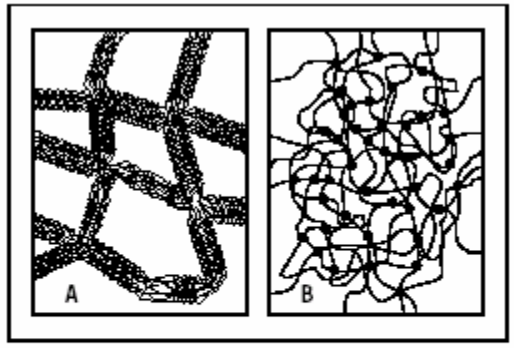
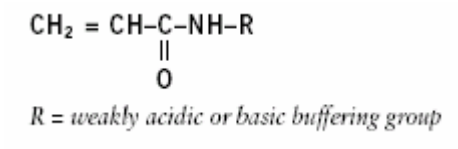
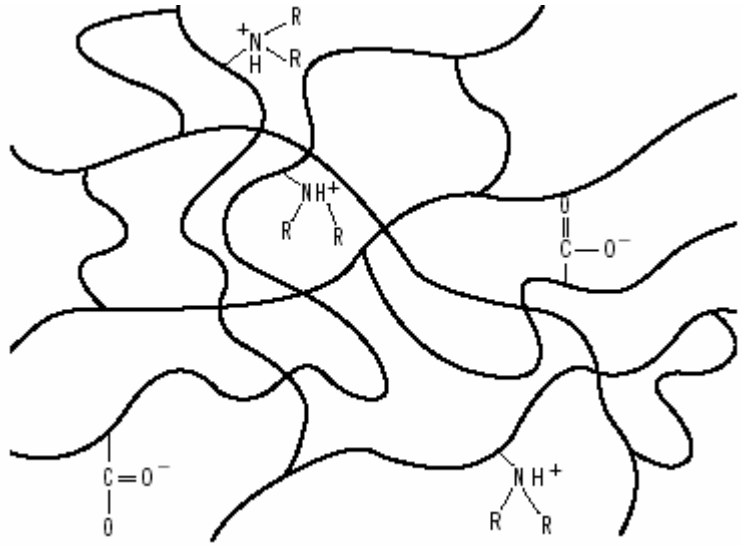


Fig 1.5. Agarose and acrylamide gels. (A) Agarose gels form by noncovalent hydrogen and hydrophobic bonds between long sugar polymers. (B) Acrylamide gels have covalent cross-links (•) between polymer strands.

1D- IEF

- Isoelectric Focussing (IEF)
  - Immobilized Gradient:
  - An immobilized pH gradient is formed along a slab of polyacrylamide gel by polymerizing a gradient mixture of acidic and basic acrylamido buffers with acrylamide and bisacrylamide.
  - The gradient is stable over time.

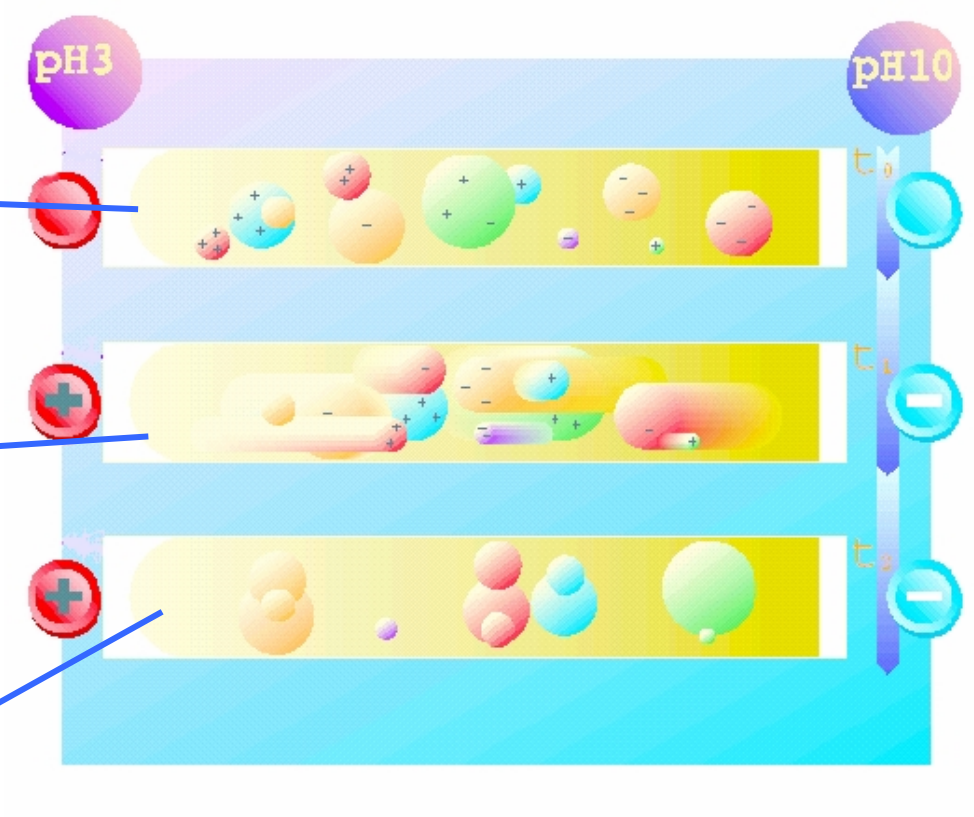




Immobiline matrix

1D- IEF

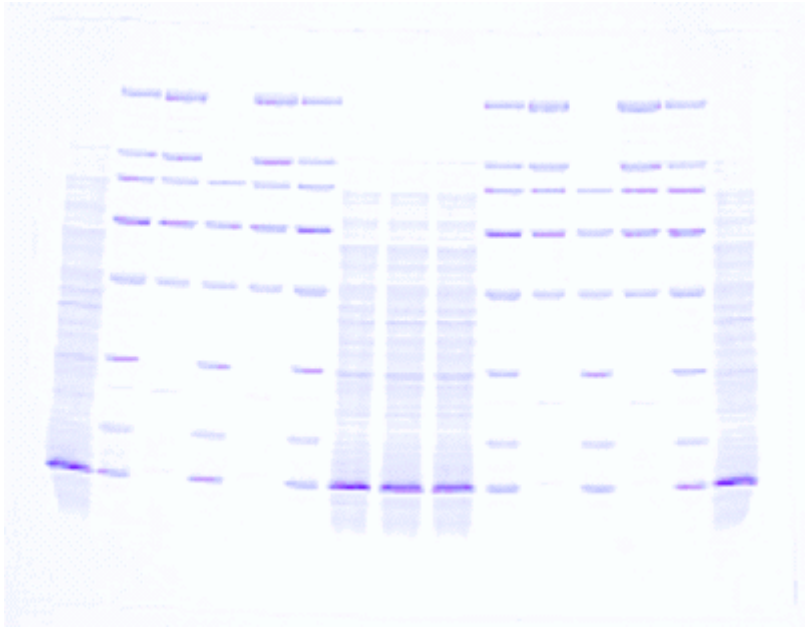
- Theory
  - A protein mixture is absorbed into the gel.
  - An electric field is then applied across the gel.
  - Proteins in regions below or above their pI migrate to the cathode or anode respectively.
  - Migration rate slows as the proteins approach the region where pH = pI
  - Protein migration ceases when pH = pI.
  - Note: proteins remain in the native state throughout the process



1D- IEF

Practice

- Preparative and analytical polyacrylamide and agarose gels can be readily cast in the laboratory for a variety of pH ranges.
- Gels are typically cast in sizes of 10 X 8 cm to 23 X 20 cm and in a variety of thicknesses.
- Precast Immobilized and ampholyte-containing gels are readily available in a variety of pH ranges.
- Precast sizes range from 0.5 X 6 cm strips for single samples to 12.5 X 24.5 cm large format for multiple samples.
- Precast gels are available in ultrathin formats, providing a high degree of resolution.

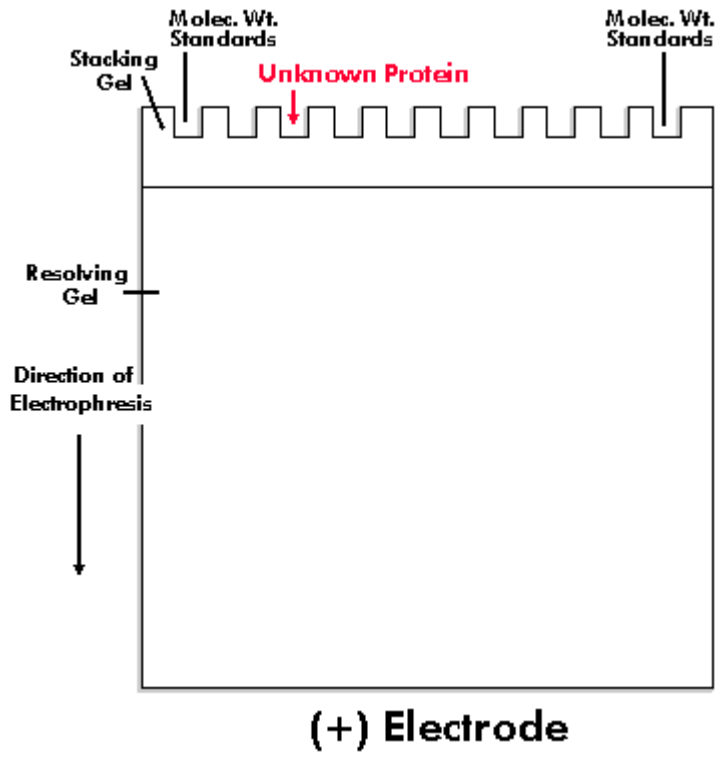


Coomassie blue stained, wide format IEF gel.

1D-SDS-PAGE

- Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
- Proteins are denatured in SDS. Negatively charged SDS coats the polypeptide evenly *circa* 1 SDS per

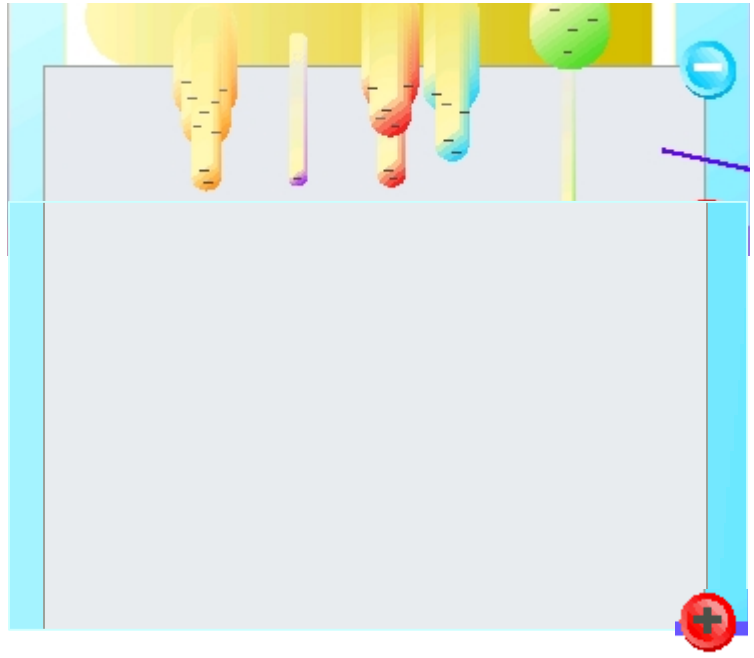
  1.5-2.0 peptide bonds
- The charge density of the denatured protein is then independent of polypeptide length.
- Protein samples are loaded in individual wells at top of polyacrylamide gel

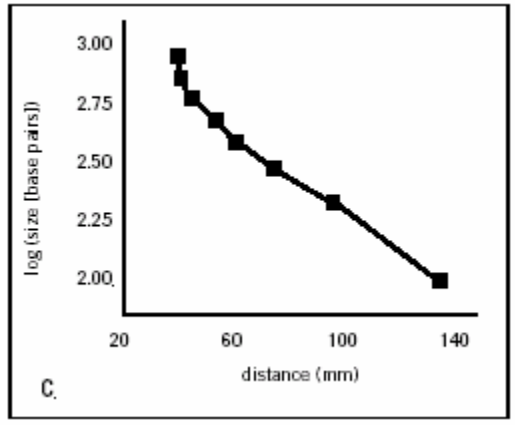


1D-SDS-PAGE

Theory

- Application of an electric field across the gel drives negatively charged SDS-coated polypeptides to the anode.
- Since the charge density is constant, the rate of migration depends on the resistive frictional force, thus short polypeptides migrate faster than long polypeptides.
- The distance traveled in a fixed time period is a log function of the molecular weight.

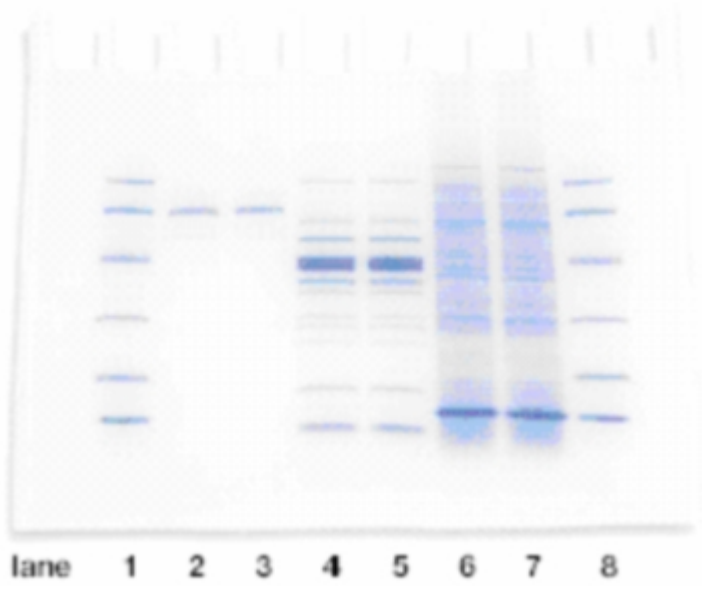




1D-SDS-PAGE

Practice

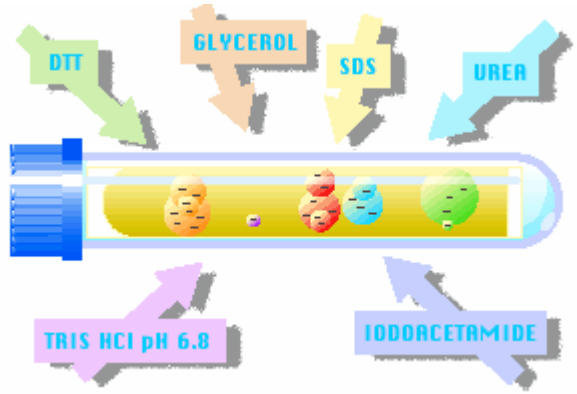
- Preparative and analytical polyacrylamide and agarose gels can be readily cast in the laboratory.
- Gels are typically cast in sizes of 10 X 8 cm to 23 X 20 cm and in a variety of thicknesses.
- Precast polyacrylamide gels are readily available in a wide range of sizes and thicknesses.



Coomassie blue stained, SDS-PAGE gel.

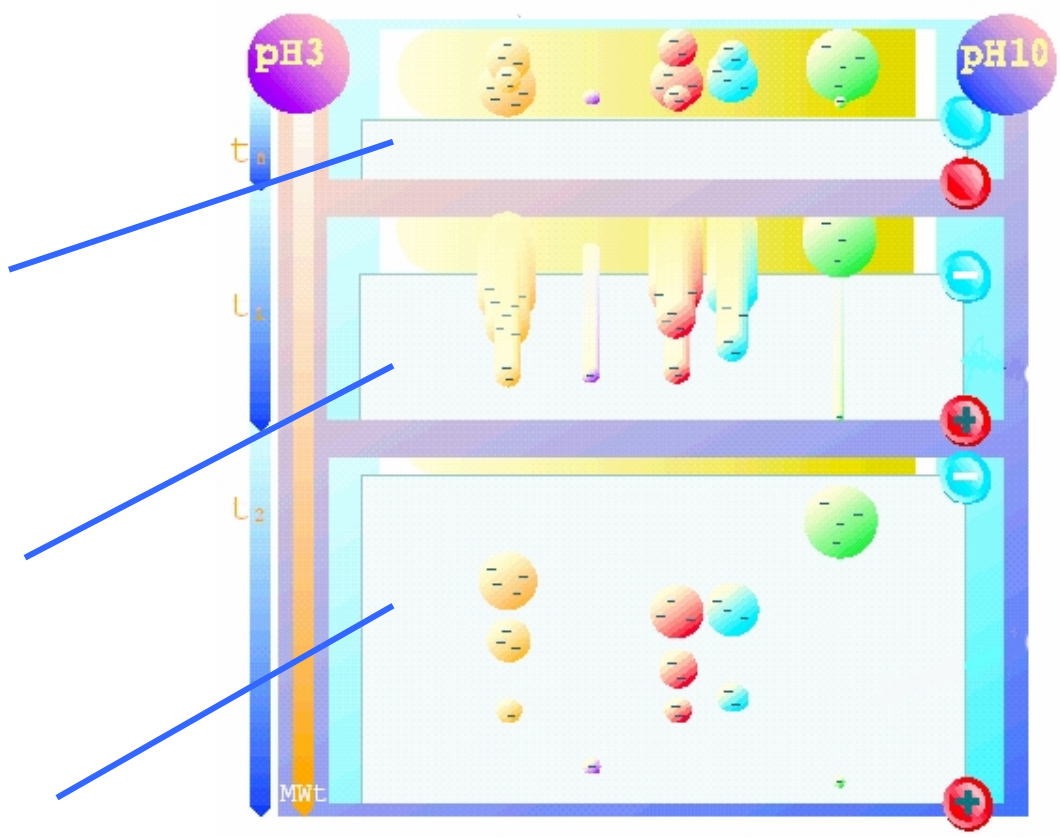
2D Gel Electrophoresis

- 2D Gel Electrophoresis Combines IEF and SDS-PAGE Separations into a Single Gel Slab.
- Proteins are first separated by IEF using narrow, immobilized gel strips (IPG).
- The IPG strips are then processed to facilitate denaturation of the focussed proteins and reductive methylation of disulfide bonds.



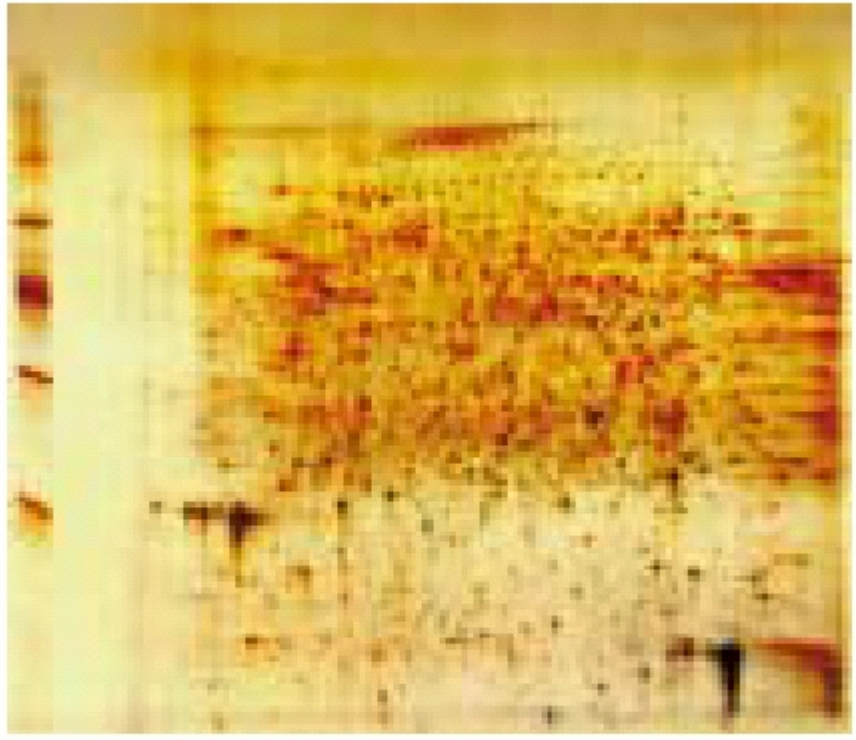
2D Gel Electrophoresis

- The second dimension involves SDS-PAGE
- The processed IPG strips are placed on top of a SDS-PAGE gel.
- The two gels are then physically and electrically connected by casting agarose over both.
- An electric field is applied across the combination and the denatured proteins migrate from the IPG gel into the SDS-PAGE gel.
- The denatured proteins migrate through the SDS-PAGE gel as an inverse log function of molecular weight.



2D Gel Electrophoresis

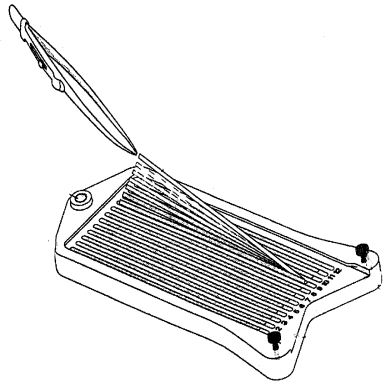
- Practice
  - Precast IPG gels are typically used for the first dimension.
  - IPGs are available in a variety of pH ranges and lengths to accommodate all standard SDS-PAGE formats
  - The second dimension polyacrylamide or agarose gels can be readily cast in the laboratory.
  - Gels are typically cast in sizes of 10 X 8 cm to 23 X 20 cm and in a variety of thicknesses.
  - Precast SDS-polyacrylamide gels are readily available in a wide range of sizes and thicknesses.

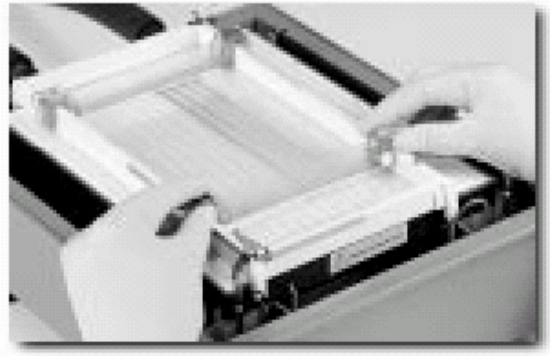


2-D electrophoresis of mouse liver proteins using 180 mm IPG strips and horizontal large scale SDS-PAGE gel, silver stained.
Görg A. Biochem. Soc. Trans. 21 (1993) 130-132 With kind permission of the author.

2D Gel Procedure & timing

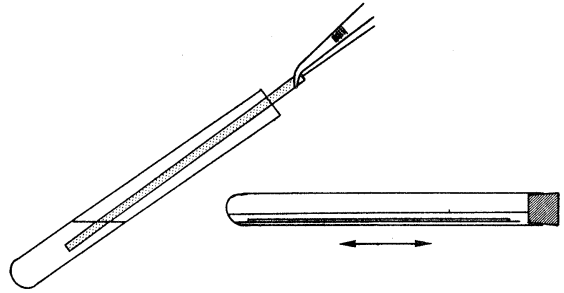
- First Dimension IEF: sample loading
- Protein samples can be loaded during the IPG rehydration step
- Up to 1 mg of total protein is dissolved in a fixed amount of hydration buffer.
- The solution is placed in a reswelling tray and a dry IPG strip placed face down on top.
- After hydration is complete, the IPG strips are placed in the flatbed apparatus face up.
- Electrode buffer wicks are added and electrodes are attached

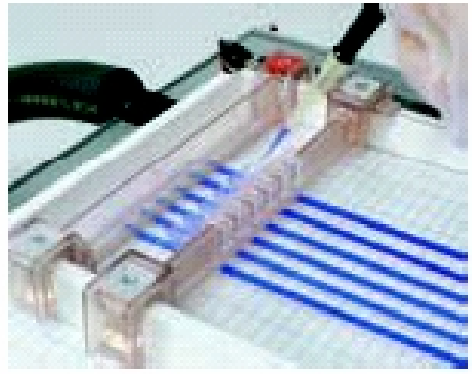




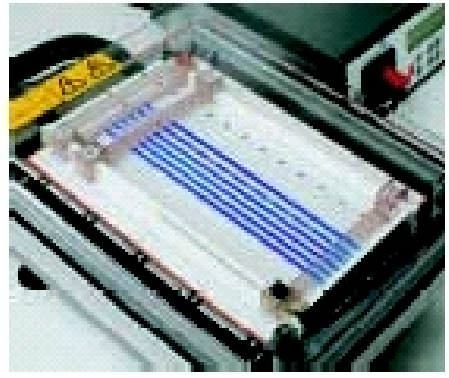
2D Gel Procedure & timing

- First Dimension IEF: sample loading
- Protein samples can also be loaded after hydration.
- IPG strips are hydrated in a tube containing the hydration buffer. The reswelling try can also be used for this purpose.
- After hydration is complete, the IPG strips are placed the flatbed apparatus face up.
- Electrode buffer wicks are added and electrodes are attached.
- A sample cup is placed on each IPG and 60-100 µg of protein can be added to each cup in a volume up to 100 µL.



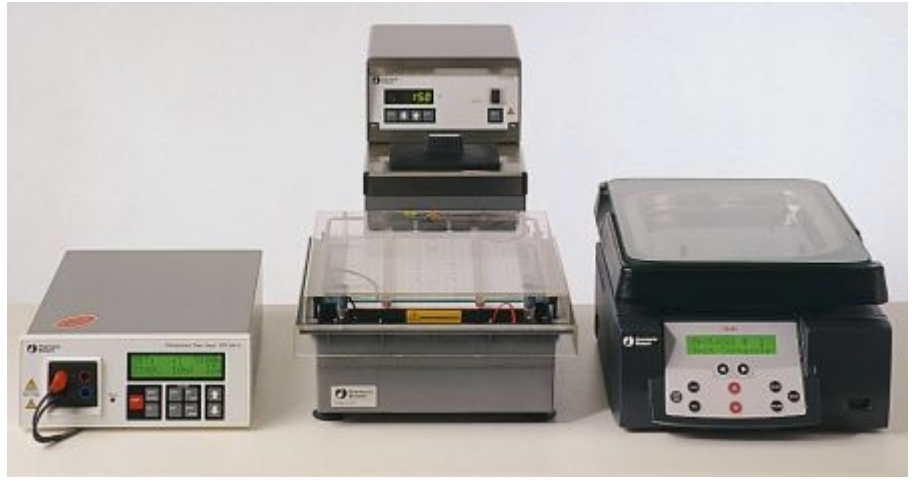


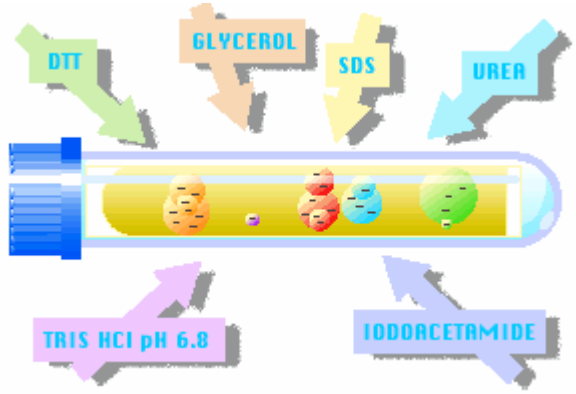
Cup loading



2D gel Procedure & timing

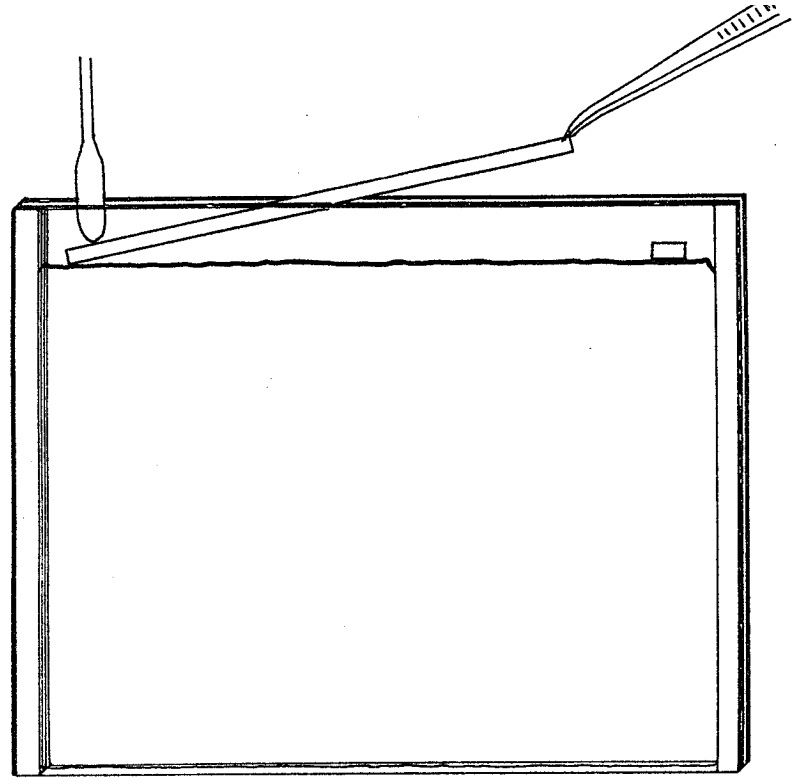
- Focussing IPG gels
  - The loaded IPGs are covered with mineral oil to help facilitate even cooling and to prevent the intrusion of carbon dioxide.
  - The apparatus is cooled to 20 ºC with an external chiller.
  - Depending on the gel length and pH range, the gels are focussed for 2000 to 25,000 Vhr.
  - Voltage is applied in steps for specific time periods for a total of 2 to 12 hrs. Focussing may be conveniently performed over night. IPG gels cannot be overfocussed.
  - Focussed gels are processed for 1-1.5 hrs (reduction, 30-45 min; alkylation, 30-45 min)





2D gel Procedure & timing

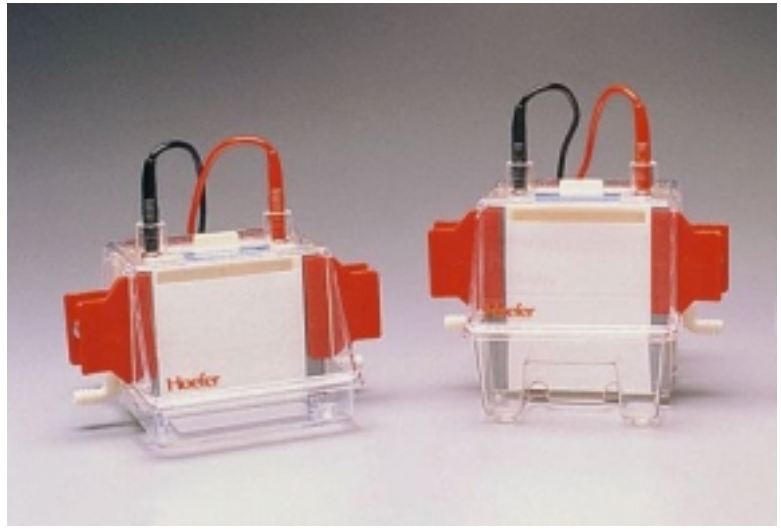
- Adding IEF to SDS-PAGE
  - Molecular weight standards can be applied to a section of filter paper and placed at one end of the SDS-PAGE gel.
  - Processed IPG strips are placed on top of SDS-PAGE gels, within the same plane.
  - The two gels are connected physically and electrically by filling the region between the top of the SDS-PAGE gel and the top of the glass plates with melted agarose.

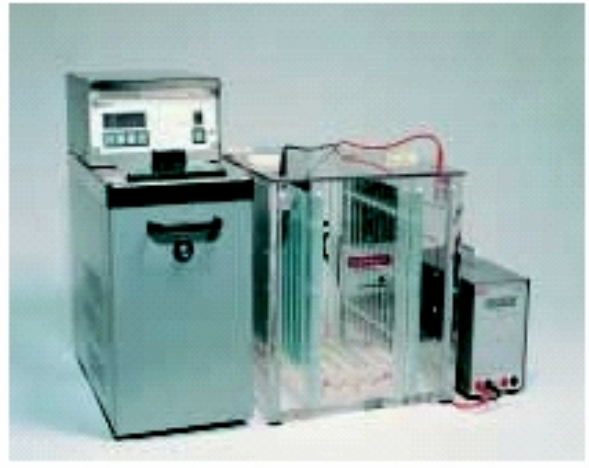


2D gel Procedure & timing

Running the Second Dimension

- Gels are loaded into a vertical electrophoresis apparatus and typically cooled in the 15-20 °C range.
- Depending on the gel size and duration of electrophoresis, current is applied at 15 mA to 45 mA per gel.
- Mini gels take about 2 hours to run, whereas large format (18 X 20 cm) gels take about 6 hours.

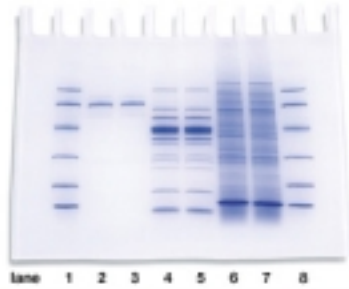




The complete solution for SDS PAGE including MultiTemp III, Hoefer DALT System and power supply EPS 2A200.

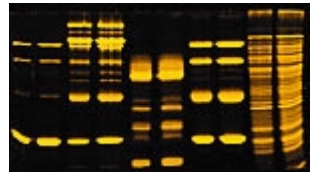
2D gel Procedure & timing

- Fixing & staining
  - Gels are “fixed” prior to staining to ensure that proteins remain denatured throughout the process.
  - The composition of fixing solutions are dependent on the stain used and the thickness of the gel. Gels are usually soaked in the fixative for 1 hr.
  - Proteins are usually stained with with either Coomassie Blue, silver, or SYPRO (fluorescent) stains. Each has its own advantages and disadvantages.



Coomassie

Silver



SYPRO orange

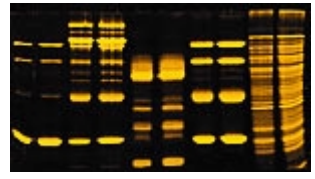
2D gel Procedure & timing

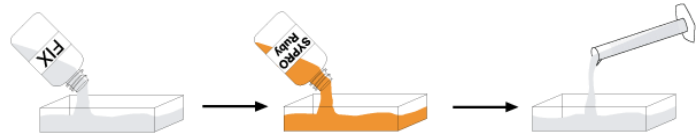
Staining

- Silver staining is a complex process, but produces an excellent, long-lasting record. Normal silver staining is not mass spec compatible!
- Coomassie and SYPRO staining are similar in complexity and timing. Depending on the thickness of the gel, Staining is accomplished by soaking the gel in stain anywhere from a few hours to overnight. Removal of residual stain (destaining) takes about 30 min with SYPRO and up to overnight with Coomassie.
- Coomassie staining provides a reasonably permanent record, whereas SYPRO stain fades with time after a few hours. Gels may be restained with SYPRO, however.

Step	Solution	Processing time
1	Fixing solution	30 min
2	Sensitising solution	30 min
3	Washing in destilled water	3 × 5 min
4	Silver solution	20 min
5	Washing in destilled water	2 × 1 min
6	Developing solution	2 – 5 min
7	Stop solution	10 min
8	Washing in destilled water	3 × 5 min
9	Preserving solution (gels on plastic backing)	20 min

Silver staining





SYPRO Staining

Documentation & Quantitation

- Sensitivity
  - SYPRO stains and silver stain have about the same sensitivity. One nanogram sensitivity is typical.
  - Coomassie staining is about 1/50th less sensitive.

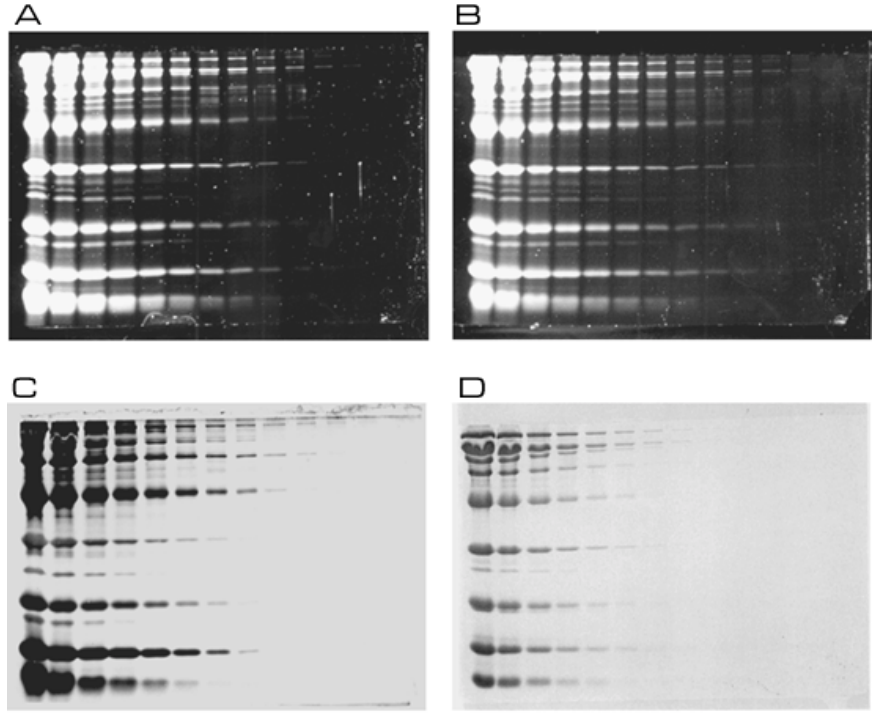


Figure 9.5 Comparison of the sensitivity achieved with SYPRO, silver and Coomassie brilliant blue stains. Identical SDS-polyacrylamide gels were stained with **A)** SYPRO Orange protein gel stain (S-6650), **B)** SYPRO Red protein gel stain (S-6653), **C)** silver stain and **D)** Coomassie brilliant blue stain, according to standard protocols. The SYPRO dye–stained gels were photographed using 300 nm transillumination, a SYPRO protein gel stain photographic filter (S-6656) and Polaroid 667 black-and-white print film. The silver- and Coomassie brilliant blue–stained gels were photographed with transmitted white light and Polaroid 667 black-and-white print film; no photographic filter was used.

Documentation & Quantitation

Linear Range

- Reliable quantitation of gel spots and bands requires a linear response of stain intensity to protein concentration.
- SYPRO stains have the largest linear dynamic range and silver the lowest. Coomassie is somewhere in the middle.
- SYPRO stains are less sensitive to protein composition than Coomassie stains.

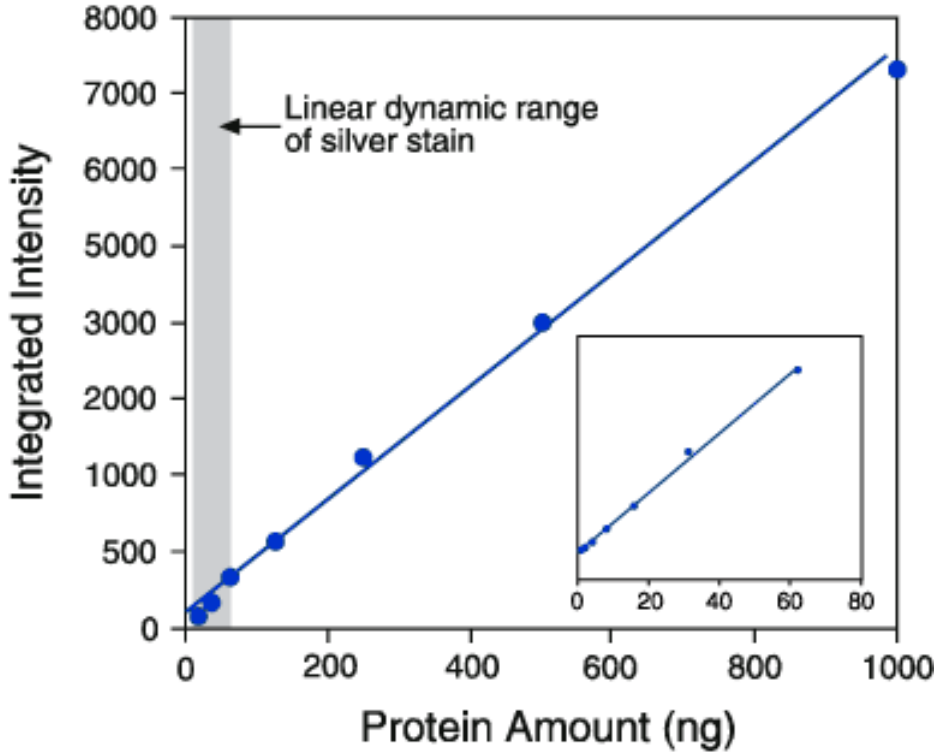


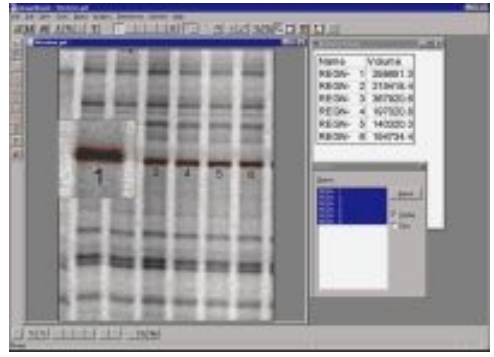
Figure 9.14 Amounts of carbonic anhydrase ranging from 1 ng to 1000 ng were separated by SDS-PAGE and stained with SYPRO Ruby protein gel stain (S-12000, S-12001, S-21900). The inset shows the excellent linearity in the lower part of the range from 1 ng to 60 ng protein. Staining intensities were quantitated using the Bio-Rad Molecular Imager FX System. For comparison, the gray band shows the linear range for the same protein detected with silver staining.

Documentation & Quantitation

Documentation

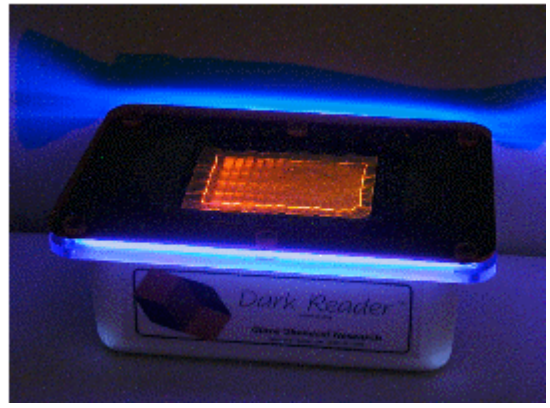
- Experimental results are normally documented by photography, by digital scanning, or by drying gels.
- Although standard photography was the mainstay for many years, it has been replaced by digital scanning.
- Digital scans can be used for quantitation, provided a calibrated densitometer is used for Coomassie and silver stained gels or a fluorescence scanner for SYPRO stained gels.
- Gels may be dried down and stored at room temperature for years.

ImageScanner™ is optimized for high throughput evaluation of electrophoresis gels.



Processing Gel Plugs for MS

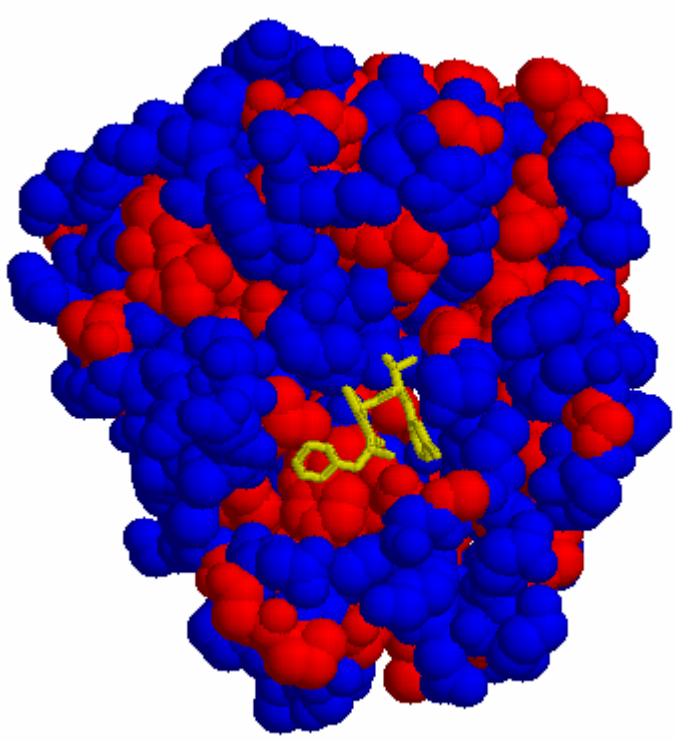
- Excising, Destaining, and Drying
  - Plugs of gel containing stained protein are cut out using a scalpel or biopsy needle.
  - Coomassie and SYPRO stains and SDS are removed by soaking plugs three times in 50% acetonitrile for 20 min each.
  - Plugs are dried by first soaking the plug in 100% acetonitrile followed by SpeedVac drying



SYPRO stained gel on fluorescent light box.

Processing Gel Plugs for MS

- In-Gel Proteolysis/ Peptide Extraction
  - Plugs are rehydrated by adding just enough concentrated trypsin solution (20 µg/mL) to rehydrate the plug.
  - 50 mM NH_4HCO_3 is added to just cover the plug and then incubated overnight at 37 ºC.
  - The buffer is removed and saved.
  - 10% formic acid is added to just cover the plug, incubated at 60 ºC for 10 min, and then sonicated for 1 hr.
  - The formic acid supernatant is combined with the incubation buffer.



bovine trypsin