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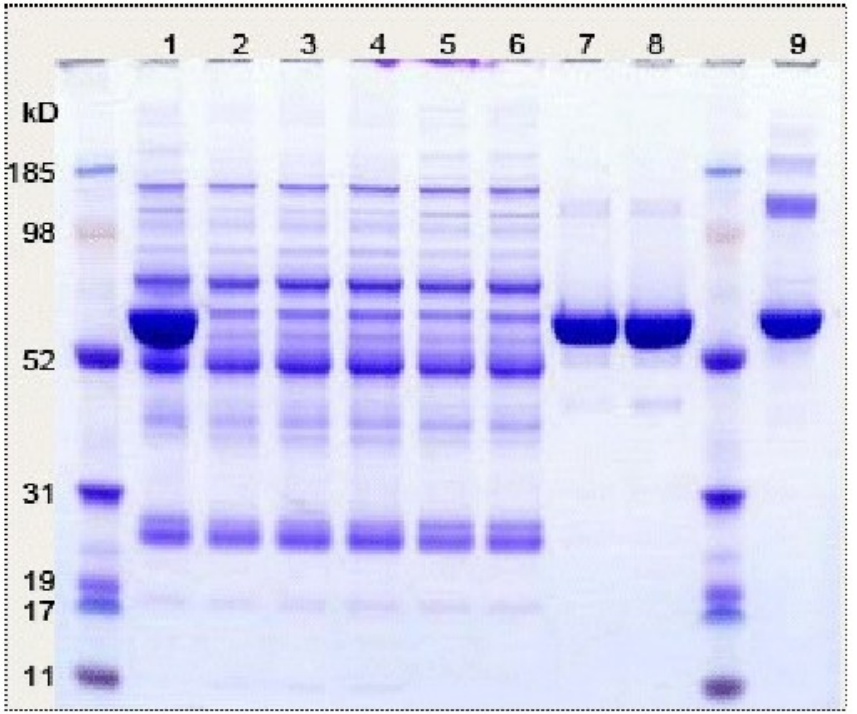
Electrophoresis (More)

Voet & Voet: Pages 144-151



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SDS-PAGE (Review)

SDS is a detergent that

1) denatures proteins (breaks apart subunits without covalent attachments eg. -S-S-)

2) binds strongly to proteins at a roughly constant ratio (~ 1 SDS/ 2 residues)

Swamps native charge of protein

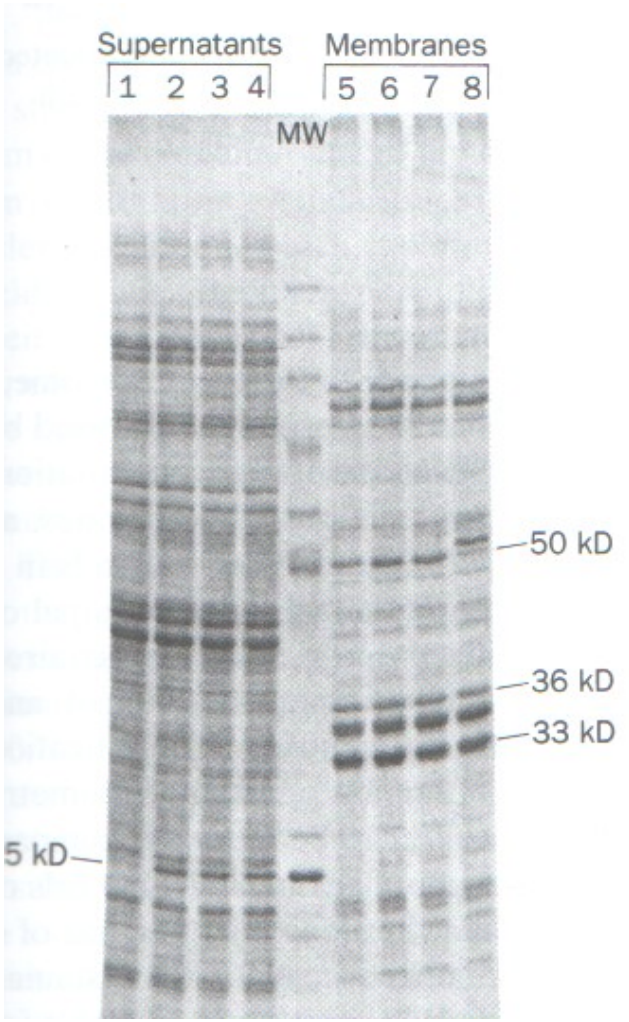
Results in ~ constant charge density AND similar shape for all proteins

Separates based upon size only

Using proteins of known size as standards, the M_w of unknown samples can be estimated

Note: If a reducing agent (eg. DTT) is included, disulfides will be reduced.

Sodium dodecyl sulfate (SDS)



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Gel Electrophoresis – Pore Size

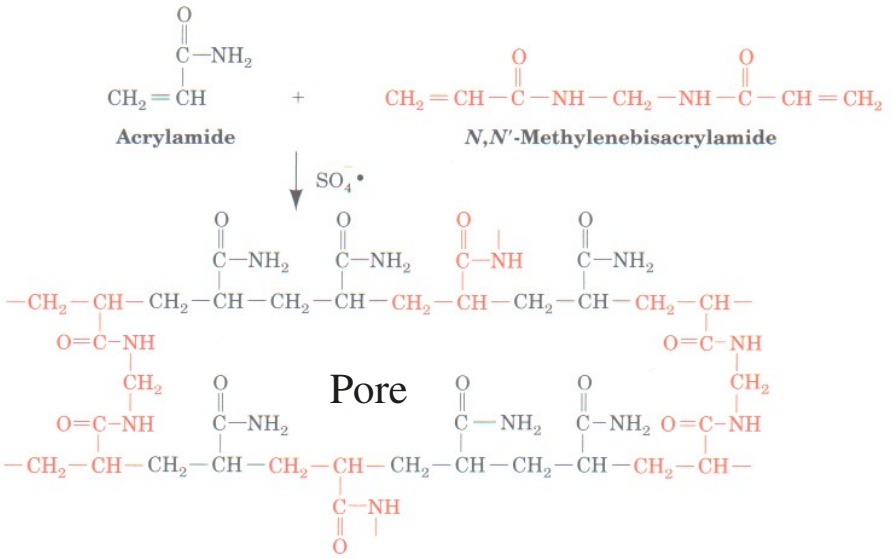
Pore size is (typically) controlled by varying the ratio of acrylamide and N,N'-methylenebisacrylamide

- Pore size decreases with increasing amounts of acrylamide (expressed as %)
- Higher % gels are more mechanical stable (and vice versa)

Each pore size (gel %) has an optimal size separation range

eg.

18% PAG	5-50 (25 optimal) kDa
15% PAG	10-70 (40 optimal) kDa
12% PAG	20-150 (85 optimal) kDa
9% PAG	30-220 (125 optimal) kDa



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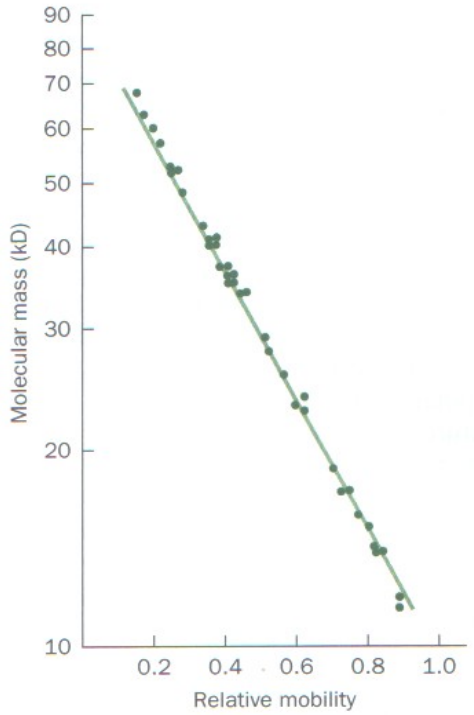
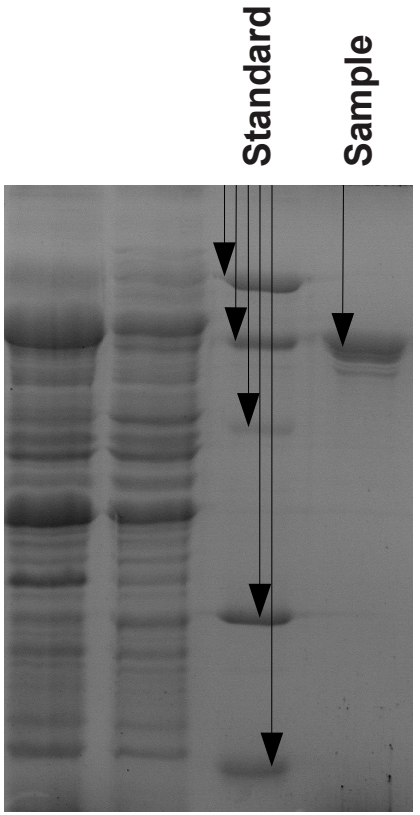
Calculating Mw

Size estimates from a standard curve of relative mobility vs Log M_w

- **Measure mobility from interface between "stacking" and "resolving" gel to middle of band**

Compare mobility of desired protein to standard curve to obtain M_w





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Western Blots

Improved detection method based upon immunoblotting

- **Up to 1000x more sensitive than Coomassie Brilliant Blue Stain**
- **Detect as little as 0.1 ng (eg. < 10 fM) using chemiluminescense (described below) or radioactivity**

Requires additional steps:

1) **transfer (blot) proteins from gel to membrane (nitrocellulose or PVDF)**
2) **Block unoccupied binding sites on membrane**
3) **Incubate with Ab (primary) that specifically binds protein of interest**
4) **Wash and incubate enzyme linked Ab; (secondary) that binds primary Ab**
5) **Assay enzyme linked Ab with chemiluminescence reaction**

Detection is not solely based upon sample quantity

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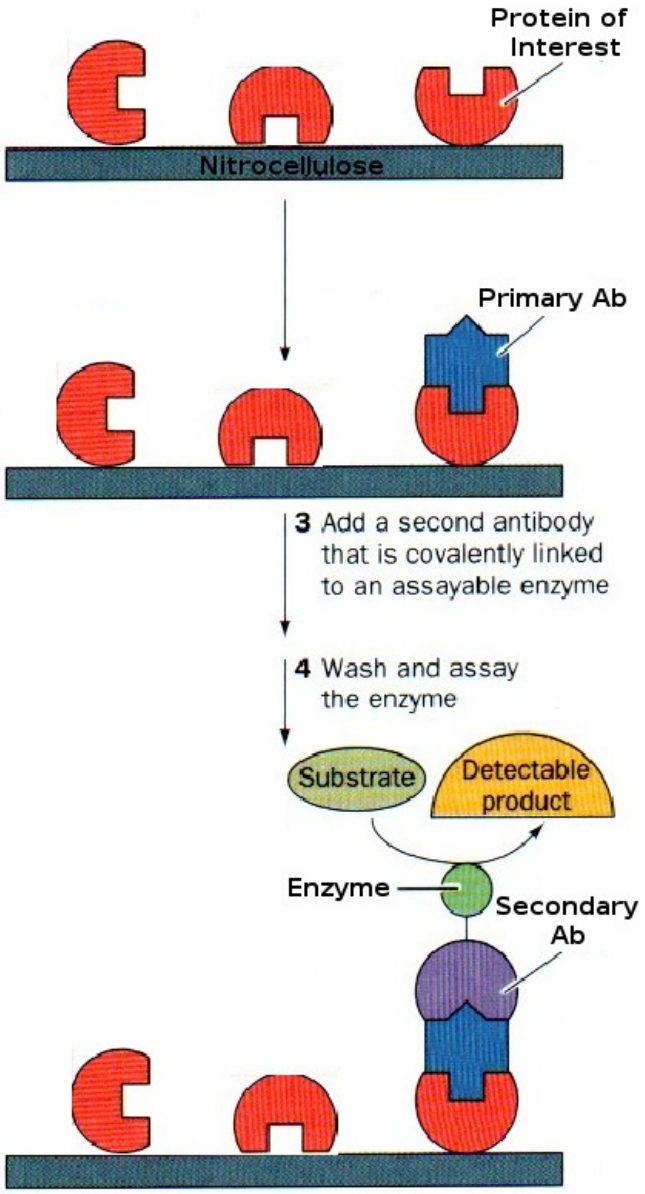
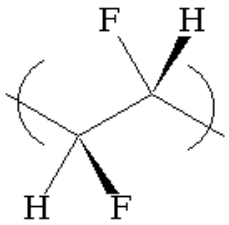
Western Blot Membranes

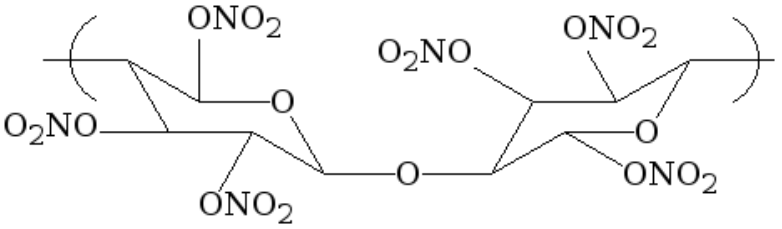
Nitrocellulose and PVDF (polyvinyl difluoride) non-specifically bind any protein

- **Proteins are electrophoretically transferred from SDS-polyacrylamide gel to the membrane**
- **Proteins are bound in many orientations (may even denature) on the membrane**
- **Remaining protein binding sites (on membrane) are blocked with non reactive proteins (eg. Milk proteins or albumin)**

  prevents non-specific Ab binding







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Native PAGE

Native or non-denaturing PAGE can be used to study macromolecular interactions

eg. Electrophoretic mobility shift assay (EMSA) or band shift assay

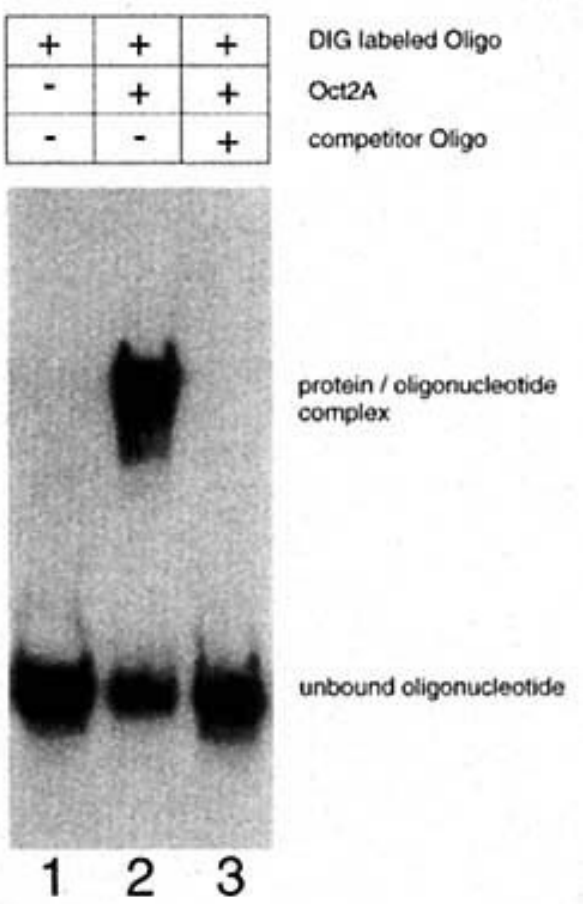
Comparison of electrophoretic mobility of a specific macromolecule:

1) Alone

2) In presence of interacting macromolecule

3) In presence of non-interacting macromolecule or competitive macromolecule

Can be used to estimated M_w though less accurate than SDS-PAGE



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More Native PAGE

In the absence of SDS, the net charge of the macromolecules under investigation is an important consideration

- **For acidic and neutral macromolecules, the normal (basic) SDS-PAGE buffers and current polarity (cathode at top of gel) allow migration of samples into gel (Note: acidic macromolecules have pI < 7)**
- **For basic macromolecules, acidic PAGE buffers and a reversed current polarity are required to allow migration of samples into the gel (Note: basic macromolecules have pI > 7)**

Typically gels are run for several hours at a relatively low voltage to prevent heating (which may lead to dissociation)

- **May even cool gel rig by placing it on ice**

Technique often (almost always) requires optimization of buffers, voltage and temperature to obtain a high quality gel

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Isoelectric Focusing (IEF)

Isoelectric focusing separates proteins based upon upon charge (pI)

- Requires a solution or gel that maintains a stable pH gradient
- Under these conditions, samples will move to a position in the pH gradient that corresponds to their isoelectric point
- Produces exceptionally sharp and narrow bands (0.01 pH units wide)

Two strategies for establishing a stable pH gradient:

1) Mix together many buffers (ampholytes) with different pK_as
   - In an electric field, each buffer in the mixture migrates to its isoelectric point establishing a gradient of pHs
2) Acrylamide derivatives of varying pK_a are mixed together and polymerized in varying ratios

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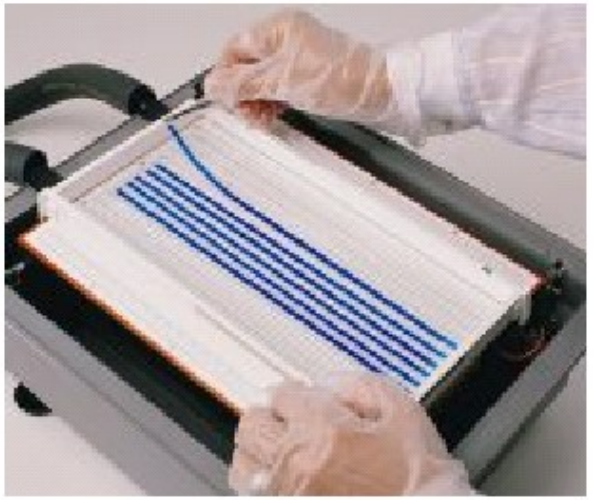
More IEF



- Requires very high voltage differences (~1000 V) and several hours to run
  - Uses very large pore (low percentage) acrylamide gel to prevent size based retardation of samples
  - Neutral denaturant (urea) that will not affect charge for denaturing IEF
  - Require lower voltage difference and longer times if you want to prevent denaturation
- Many proteins that are “pure” by SDS-PAGE are resolved into multiple components by IEF
  - Protein modifications (eg. deamination, phosphorylation, ...) can change the net charge of a protein without significantly affecting its M_w
- Often used when samples of the highest purity are required

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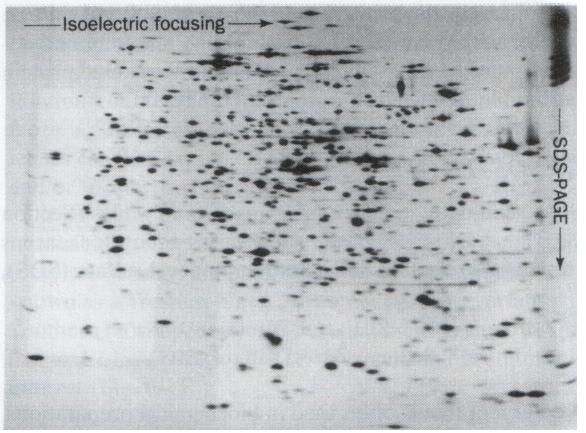
2D Gels

Combines power of IEF and SDS-PAGE on a single gel

- IEF followed by SDS-PAGE in the orthogonal direction
- Can resolve up to 5000 proteins on a single gel
- ~50% of cellular proteins

Used to compare proteins present (in a cell type) under various conditions

- eg. stressed vs unstressed, diseased vs normal, … (example of proteomics)



Individual protein bands can be cut from the gel and identified by sequencing

- typically use Biophysical Sequencing (Mass Spec)

More 2D

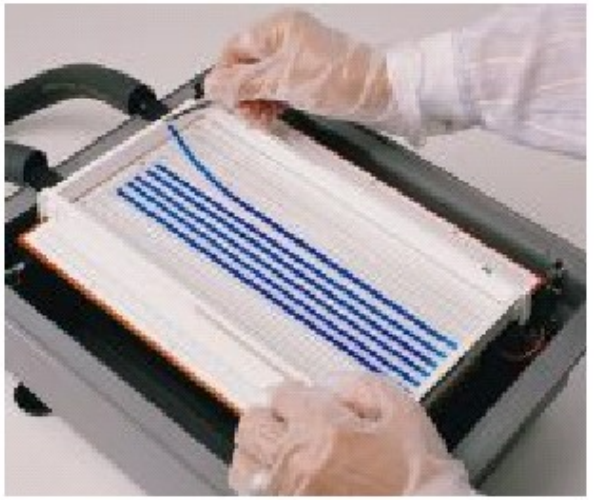
Gel is run in several steps

(1) Initially the IEF gel is run in a thin strip

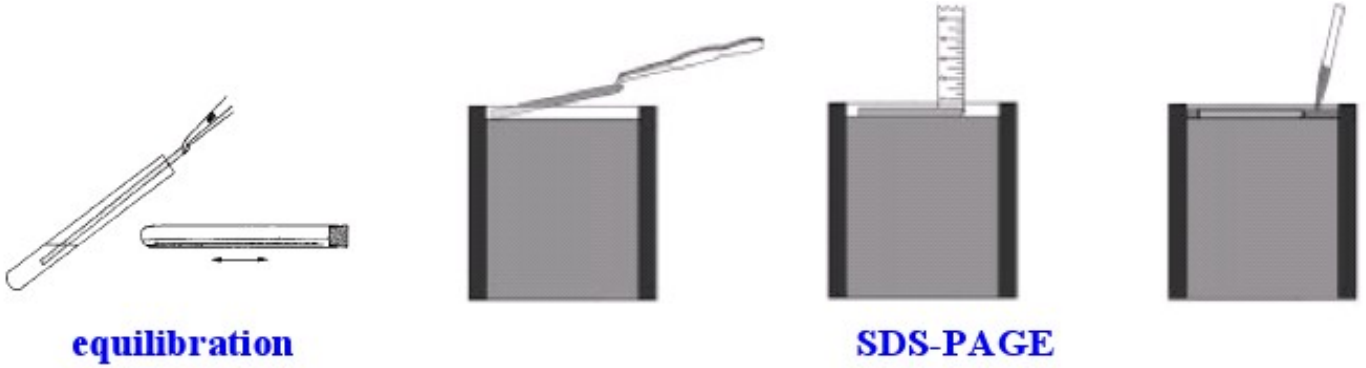
(2) IEF gel is equilibrated in SDS-PAGE buffer

(3)IEF gel strip is laid horizontally on top of an SDS-PAGE gel

(4) SDS-PAGE run in the orthogonal direction compared to the IEF gel



Note: Number of proteins visualized depends upon detection method



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Capillary Electrophoresis

- Electrophoresis in an extremely thin tube (< 0.1 mm)
  - Rapid heat dissipation allows the use of higher voltage (100-300 V cm^{-1}) and shortens separation time from an hour to minutes.
  - Small tube limits convective mixing and produces exceptional sharp peaks
  - Much lower detection limit
  - Can be automated
- Exclusively an analytical tool as you cannot load enough protein for preparative work
  - IEF, Native or SDS-PAGE separations may be carried out using capillary electrophoresis

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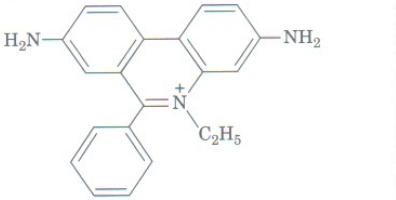
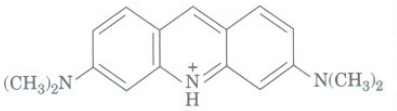
Nucleic Acids & Electrophoresis

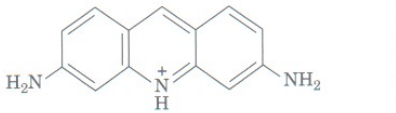
- For large macromolecules (eg. Nucleic Acids), Agarose maintains its mechanical stability better than polyacrylamide and is the gel of choice
  - Nucleic acids have a large negative charge and do not require SDS or high pH in order to migrate towards the anode
  - Detection is by intercalating agents that bind double stranded regions and fluoresce
    - Planar aromatic cations that slip in between consecutive bases in nucleic acids
  - Note: Even single stranded nucleic acids typically have small regions that are double stranded



Ethidium



Acridine orange



Proflavin

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Pulse Field Gel Electrophoresis

- Upper limit for Agarose Gel Electrophoresis is ~100 kbp (~66 MDa)
- Modification of method (Pulse Field Gel Electrophoresis) allows sample of up to 10 Mbp (~6600 MDa)
  - Method uses two sets of orthogonal electrodes
  - A pulse of current is sent through first one and then the second set of electrodes (alternating)
- Nucleic Acid must reorient (rotate by 90°) each time the current switches between electrodes in order to pass through pores
  - Time to reorient depends upon size