Visualizing DNA (and RNA, protein): non-specific (bulk) detection methods

I. Quantitation of nucleic acids (chemical properties: bases, dye binding)

II. Electrophoresis (PO_4^- groups, size)

III. Visualizing macromolecules (e.g. dye binding)

Note:
Many protocols can be found at http://openwetware.org

Guide to readings: DNA & protein visualization (non-specific)

1) *3 MC4 DNA quantitation*. Discussion of UV spectroscopy, and stain-based methods for DNA analysis.

2) <u>Electrophoresis to separate DNA and proteins</u>

- *4 MC4 Agarose electrophoresis*. Details of agarose gels for DNA analysis.
- *5 MC4 Polyacryamide gel electrophoresis (PAGE).* Protocol for DNA PAGE gels.
- *6 MC4 SDS-PAGE for proteins.* Protocol for separation of proteins on polyacrylamide gels.

3) <u>Staining to reveal biomolecules</u>

- *7 MC4 vis DNA.* Stains for DNA visualization. Also, biotinylation, and the use of magnetic beads for DNA
- *8 MC4 vis protein.* Stains for protein visualization.

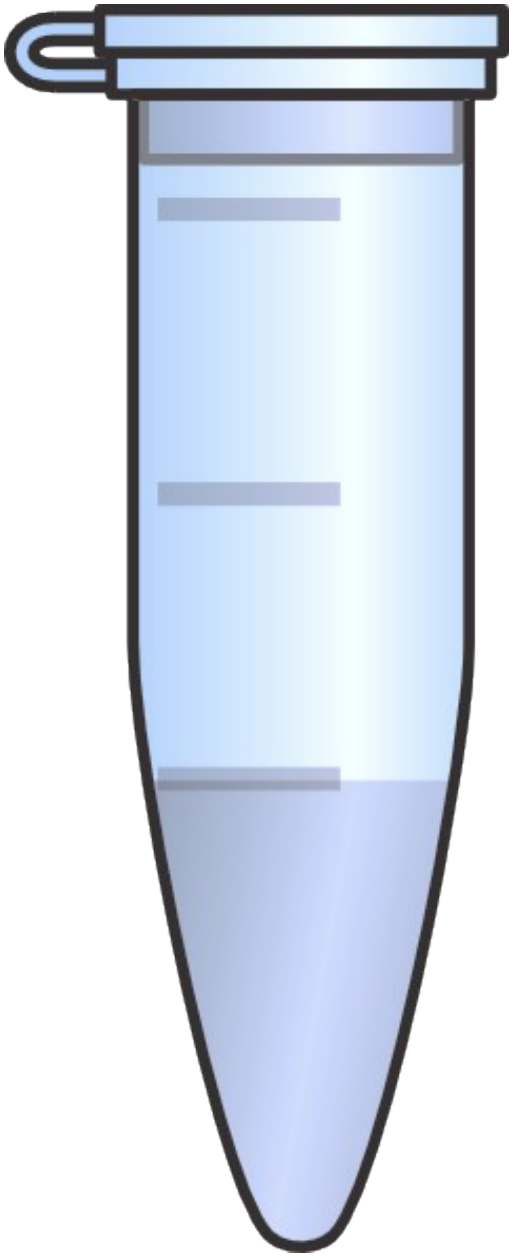
You just purified some genomic DNA

It’s dissolved in water.

How much DNA is in there?

Do you have enough to proceed with the next step in the protocol?

Do you have the right DNA?



DNA absorbs short wavelength light (260 nm). The more DNA in a solution, the less light gets through

The Beer-Lambert law:

$$I = I_o 10^{-\varepsilon dc}$$

How much light gets through a solution depends on what's in it and how much there is

I = intensity of transmitted light

I_o = intensity of incident light

ε = molar extinction coefficient

d = optical path length

c = concentration of absorbing material

DNA and RNA have specific ε s

The Beer-Lambert law

$$I = I_o 10^{-\varepsilon dc}$$

*A*bsorbance: $\underline{A}$ measured by a spectrophotometer is $\underline{\log I/I_o}$

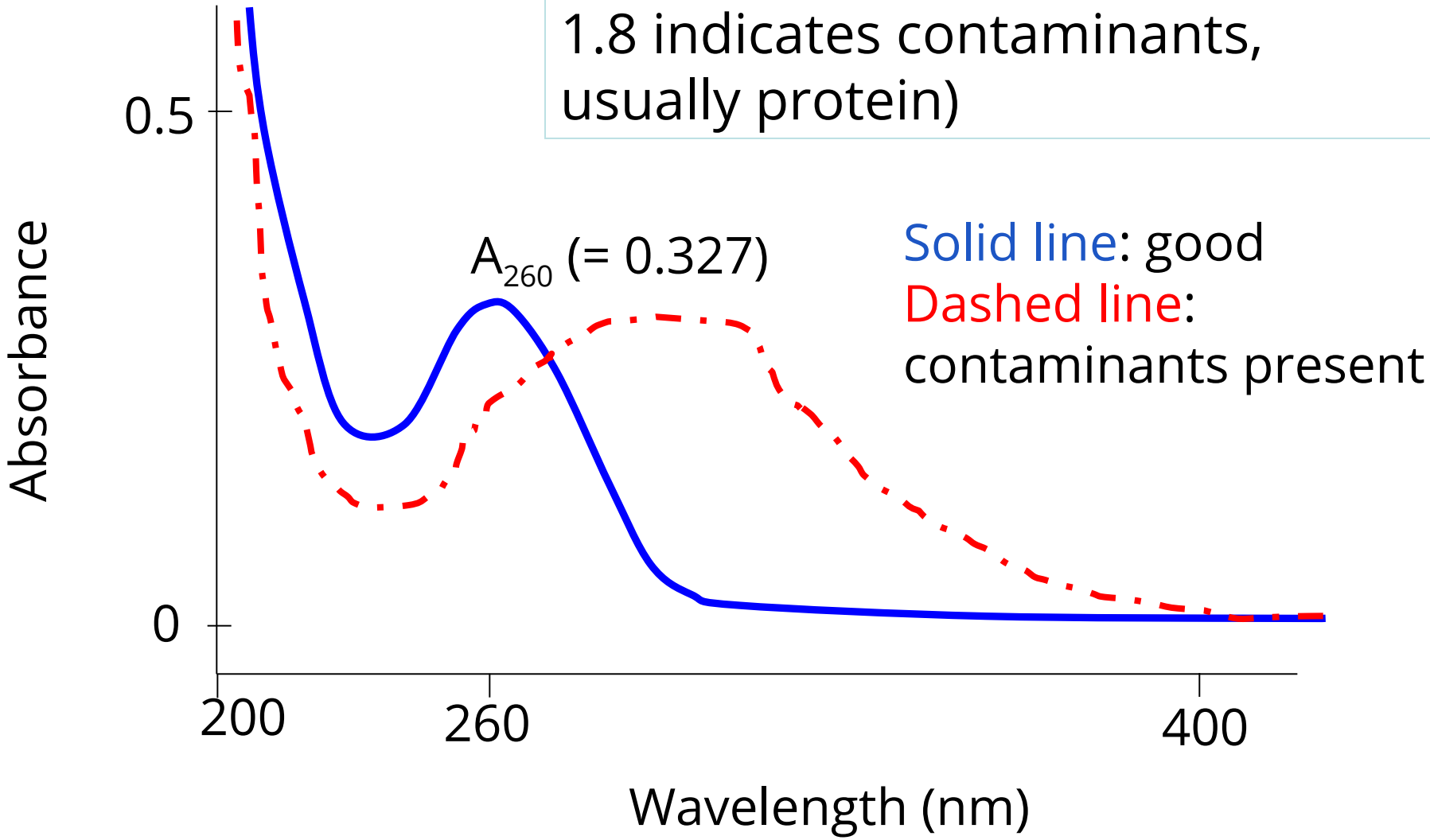
A is called optical density (OD) when the path length *d* is 1 cm

If you know the ε of the substance, the absorbance of a solution will tell you its concentration:

$$OD_\lambda = \varepsilon c$$

A " scan" (multiple wavelength absorbance measurement) of a DNA sample

A_{260}/A_{280}: 1.8 is good (lower than 1.8 indicates contaminants, usually protein)



Quantitation of DNA: UV absorbance at 260 nm

- Nucleic acids: the aromatic bases have a characteristic absorbance maximum at around 260 nanometers
- Sample must be pure for accurate measurements (RNA, EDTA, phenol, etc. absorb at 260 nm)

- For a reading of 1.0 A_{260} (1 cm light path)
  - DNA (double stranded) is 50 micrograms/milliliter
  - DNA (single stranded) 33 micrograms/milliliter
  - RNA is 40 micrograms/milliliter
  - http://nebiocalculator.neb.com/#!/od260

SI Unit prefixes and symbols

Factor	Prefix	Symbol	Example
$1{,}000{,}000{,}000 = 10^{9}$	giga	G	1 gigameter (Gm) $= 10^{9}$ m
$1{,}000{,}000 = 10^{6}$	mega	M	1 megameter (Mm) $= 10^{6}$ m
$1{,}000 = 10^{3}$	kilo	k	1 kilogram (kg) $= 10^{3}$ g
$100 = 10^{2}$	hecto	h	1 hectogram (hg) $= 100$ g
$10 = 10^{1}$	deka	da	1 dekagram (dag) $= 10$ g
$0.1 = 10^{-1}$	deci	d	1 decimeter (dm) $= 0.1$ m
$0.01 = 10^{-2}$	centi	c	1 centimeter (cm) $= 0.01$ m
$0.001 = 10^{-3}$	milli	m	1 milligram (mg) $= 0.001$ g
$^{*}0.000\,001 = 10^{-6}$	micro	μ	1 micrometer (μm) $= 10^{-6}$ m
$^{*}0.000\,000\,001 = 10^{-9}$	nano	n	1 nanosecond (ns) $= 10^{-9}$ s
$^{*}0.000\,000\,000\,001 = 10^{-12}$	pico	p	1 picosecond (ps) $= 10^{-12}$ s
	10^{-15} femto	f	1 femtomole (fmol)=10^{-15} mole

* For very small numbers, it is becoming common in scientific work to leave a thin space every three digits to the right of the decimal point.

10^{0} = the unit of measurement: -mole, -meter, -gram, etc.

Convert A_{260}:

- to micrograms/ml
- to molar concentration

Example:

sample of 250 bp fragment of double stranded DNA

A_{260} = 0.327

What is the DNA concentration?
(1.0 A_{260} = 50 micrograms/ml double stranded DNA)

DNA conc. = 0.327 x 50 = 16.35 micrograms/ml

Molar concentration of a DNA solution

Average molecular weight (MW) per base pair = 650

250 base pair DNA MW = 1.6×10^{5}, so

So 1.6×10^{5} grams of this DNA fragment per mole

Solve for molarity (moles/liter): 1.02×10^{-7} M

Convert to a less unwieldy notation: 102 nanomolar (nM)

Important to know how to do this calculation and conversion

Or go to: http://nebiocalculator.neb.com/#!/dsdnaamt

What is the molarity of a 16.35 microgram/ml solution of a 250 base pair DNA fragment?

16.35 micrograms	1000 ml	1 gram	1 mole
1 ml	1 L	10^6 micrograms	1.6×10^5 grams

1.02×10^{-7} molar
0.102×10^{-6} molar [0.1 micromolar (μM)]
102×10^{-9} molar [102 nanomolar (nM)]

Fluorometry: another method for quantitation of DNA

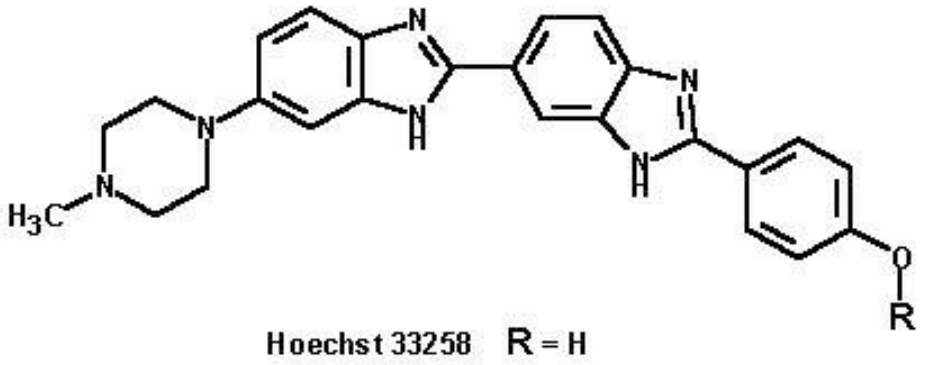
- Hoechst 33258 (a fluorescent dye) binds to DNA in the minor groove (without intercalation)

- Fluorescence increases after DNA binding

- Good for quantitation of low concentrations of DNA (10-250 ng/ml [pg/µl])

- rRNA and protein do not interfere

- Requires a fluorometer



Hoechst 33258 R = H

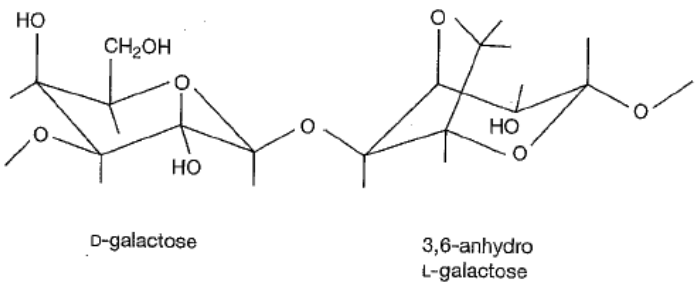
http://www.maizegdb.org/mnl/75/images/26konstantinovf1.jpg

Visualizing biomolecules: electrophoresis

- Separate biomolecules based on their size
- The separation matrix, or gel (agarose or polyacrylamide), is saturated with an electrically conductive buffer. Samples are loaded, an electric field is applied, and negatively charged biomolecules in the sample travel toward the cathode
- The choice of matrix depends mainly on the size of DNA, being analyzed
- Larger molecules travel slower through the gel matrix
- Dyes allow visual estimate of travel through the gel

Agarose gels

Agarose: a polysaccharide polymer of alternating D- and L-galactose monomers, isolated from seaweed



Agarose structure

- Pore size is defined by the agarose concentration (higher concentration, slower DNA migration overall)
- The conformation of the DNA (supercoiled, nicked circles, linear) affects the mobility of the DNA in gels
- Rate of DNA migration is affected by voltage (5 to 8 Volts/cm is considered optimal)
- Many kinds of agarose are available (variable melting temperatures, generated by differential hydroxyethylation of the agarose)

More about gels

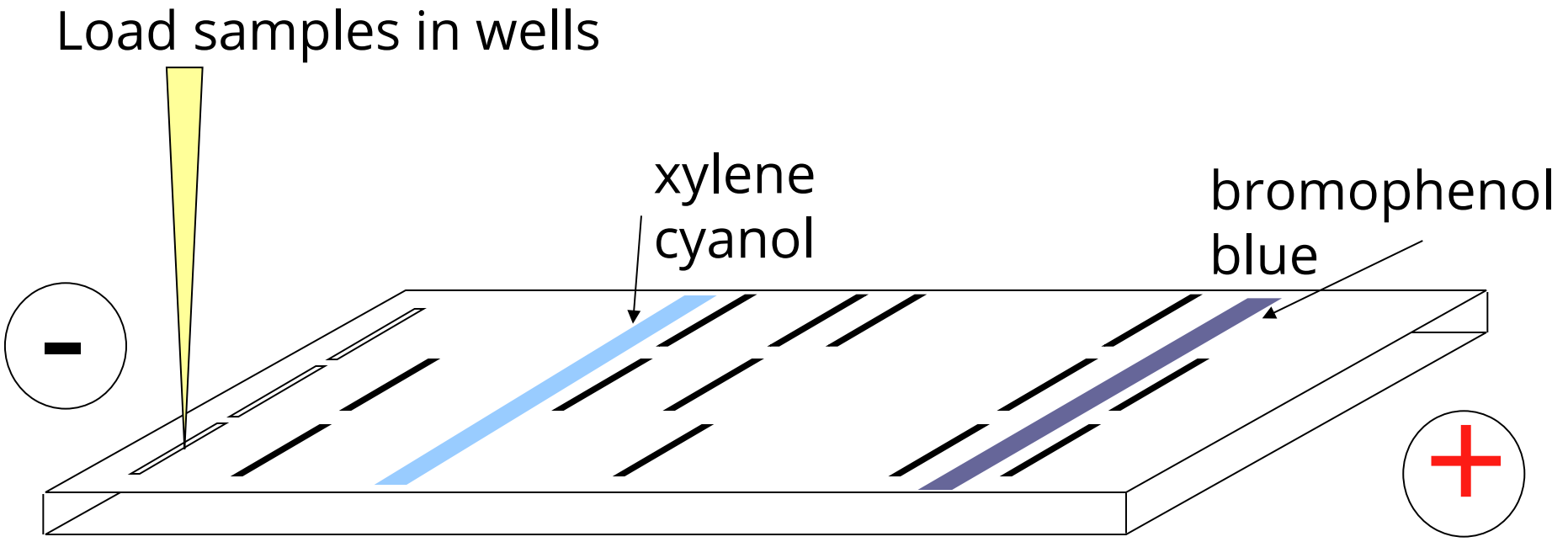
There has to be a buffer/salt (for carrying current and maintaining pH)

- TAE (Tris-acetate-EDTA): good resolution of DNA, but buffering capacity is quickly depleted
- TBE (Tris-borate-EDTA): High buffering capacity, resolution is pretty good

Use gel loading " buffers" (relatively simple)

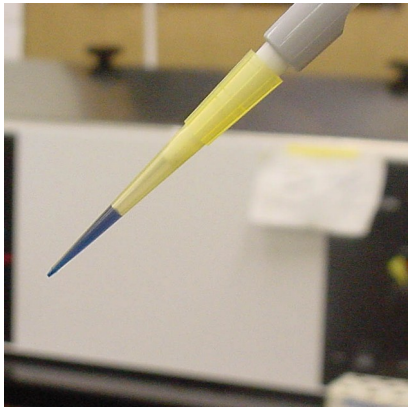
- Dense material to carry sample to bottom of wells (sucrose, glycerol, or ficoll)
- Dyes for tracking progress of electrophoresis
  - Bromophenol blue: fast migration
  - Xylene cyanol: slow migration
- Occasionally denaturant is present (formamide) for denaturing gels (e.g. sequencing gels)

Typical agarose gel

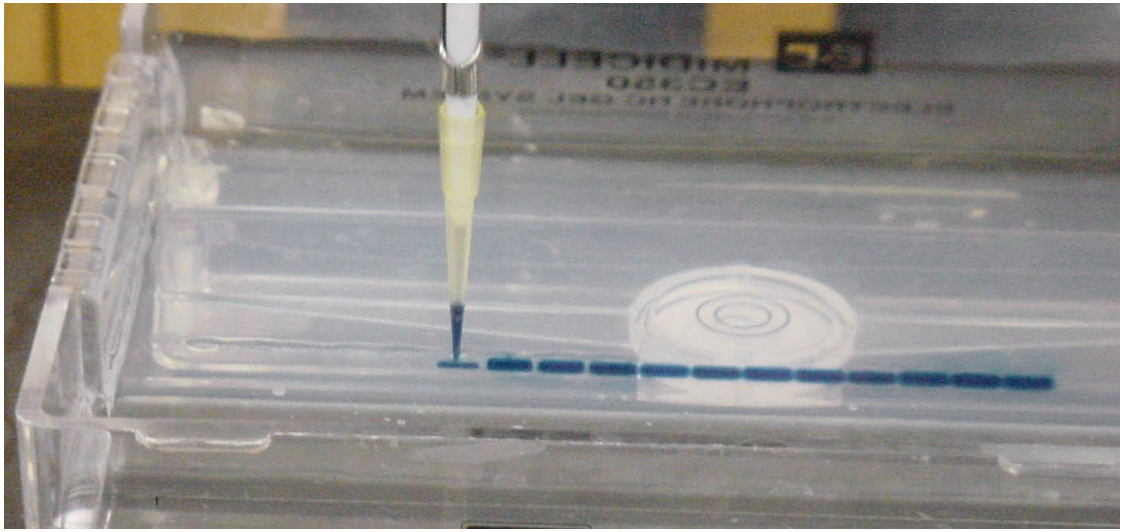


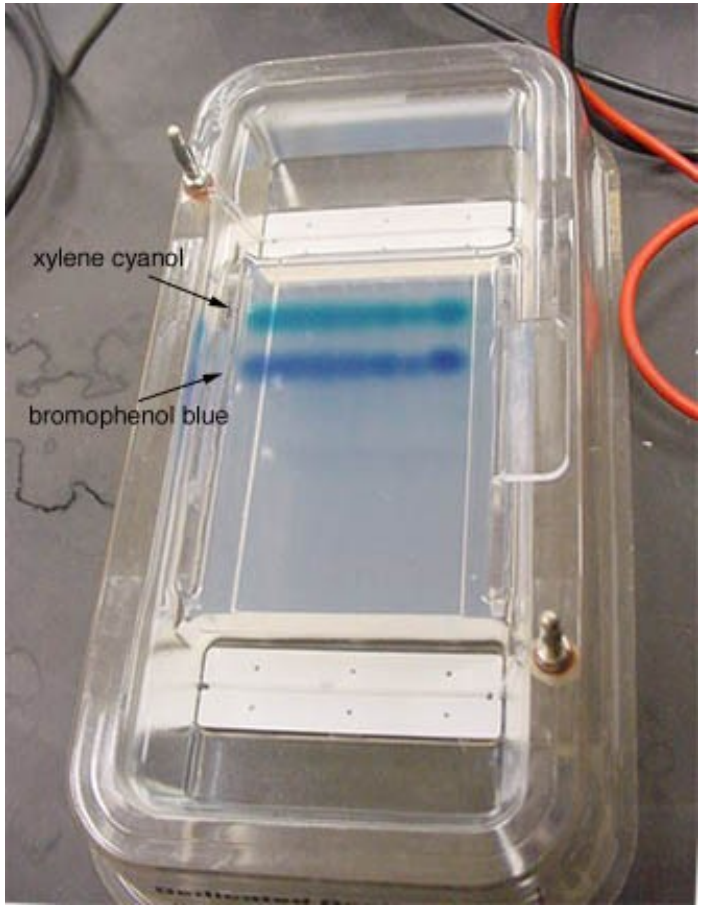
(the DNA fragments are not visible without some sort of staining)

time of electrophoresis
(progress monitored by marker dyes)



Agarose gels

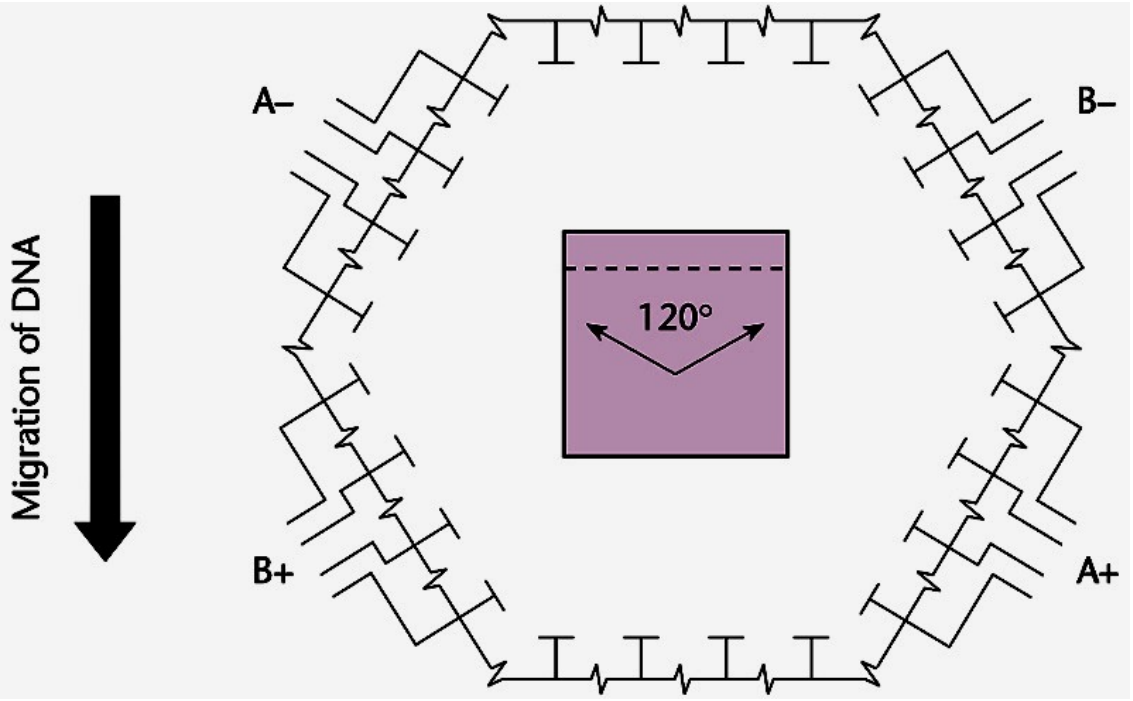




Agarose gels

Standard gels can separate DNA fragments from 100 bp to about 20,000 bp

Pulsed-field gels separate very large DNA fragments (up to 10,000,000 bp, or 10 Mb)



This apparatus induces periodic shifts in the direction of DNA migration: 120° refers to the reorientation angle (difference between orientation of electric fields A and B

Polyacrylamide gel electrophoresis

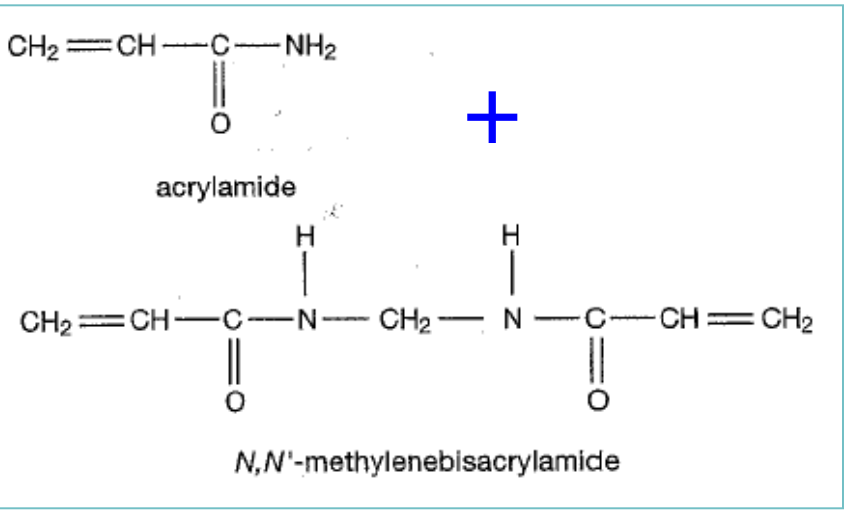
- Acrylamide monomers (toxic!) polymerized to form gel matrix
- The gel structure is held together by the cross-linker-- usually N, N'-methylenebisacrylamide ("bis" for short)
- Pore size defined by concentration of gel (total percentage) and concentration of the crosslinker (bis) relative to acrylamide monomer
- Very high resolution (better than agarose)

- Works well for smaller nucleic acids (from 6 to 1000 base pairs in length, RNA or DNA)

Polyacrylamide synthesis

Monomers → *polymer*

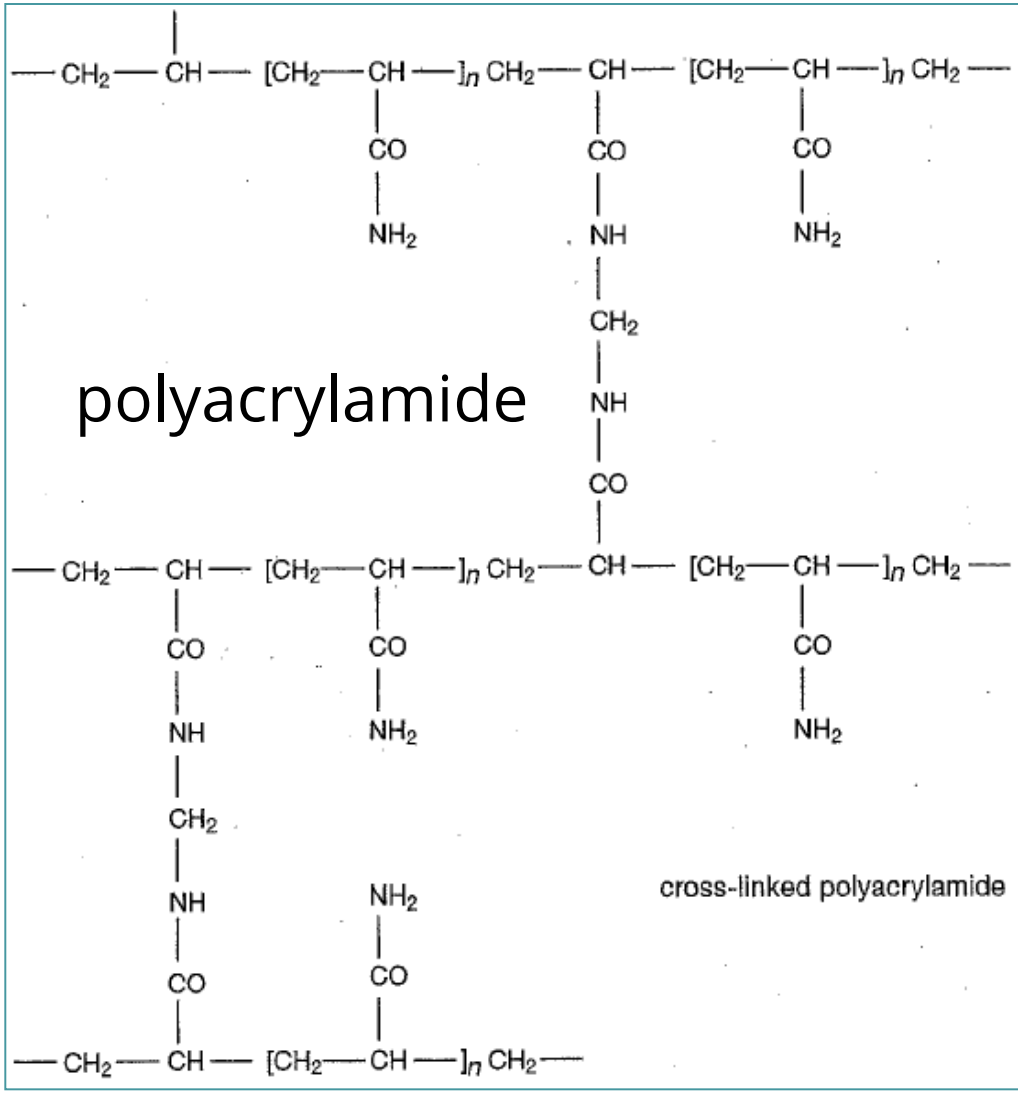
acrylamide (toxic!)



bisacrylamide

=

polyacrylamide



Recipe for a polyacrylamide gel

- Acrylamide (anywhere from 4 to 20 %, depending size of nucleic acids or proteins in the gel)
- Bis-acrylamide (the ratio of Bis to regular acrylamide is important)
- Water
- Buffer

To initiate polymerization, add

APS: Ammonium persulfate

-- generates free radicals needed for polymerization

TEMED: N,N,N’ ,N’ - tetramethylethylenediamine

-- accelerates free radical generation by APS

Polyacrylamide gel electrophoresis

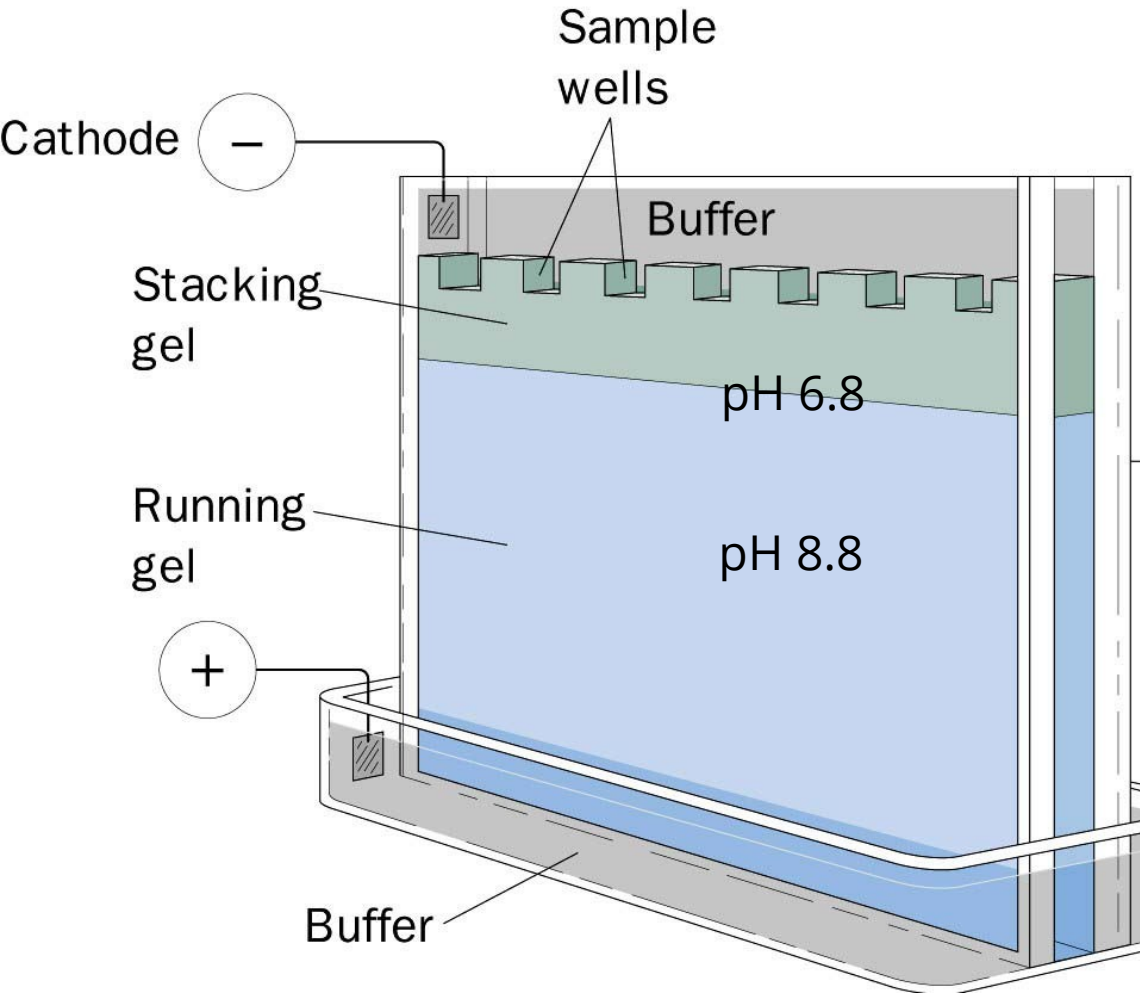
- Native gel (DNA stays double-stranded), or:

- Denaturing gel -- run in the presence of high concentrations of denaturant (usually urea. DNA runs in single stranded form (sequencing gels)

Protein electrophoresis

- Polyacrylamide gel
- The anionic detergent SDS (sodium dodecyl sulfate) is used to denature the proteins, giving each protein a “ uniform” negative charge
- Protein separation occurs as a function of size
- Discontinous Tris-Cl/glycine buffer system:
  - Stacking gel: pH 6.8, low polyacrylamide concentration, focuses proteins into thin layer (gives higher resolution upon separation)
  - Separating gel: pH 8.8, separates proteins on the basis of size

Protein gel
"SDS-PAGE"



Stacking gel

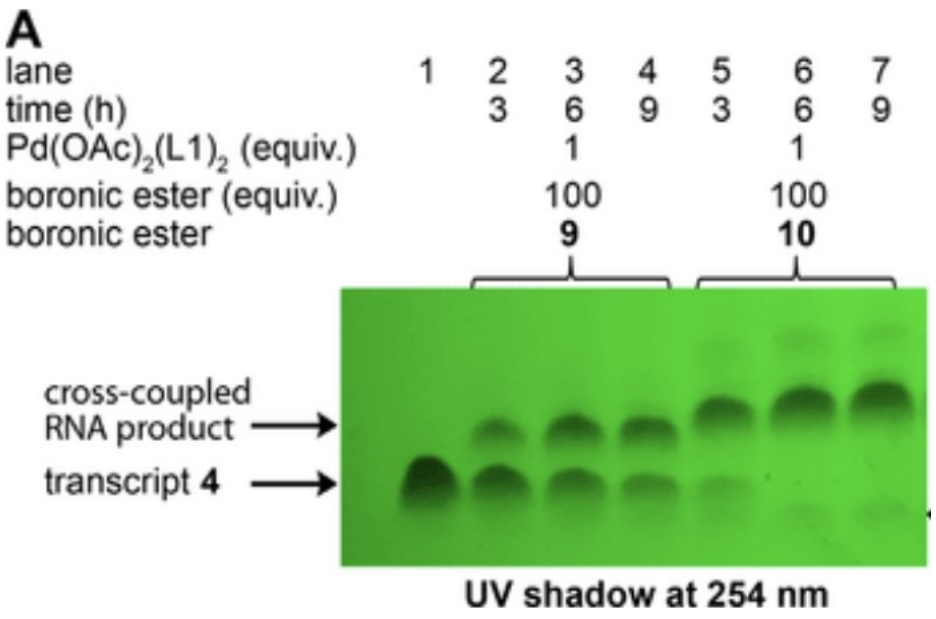
At *low pH*, glycine tends to be protonated (no negative charge), Cl^- ions form the leading edge, glycine trails, steep voltage gradient in between,

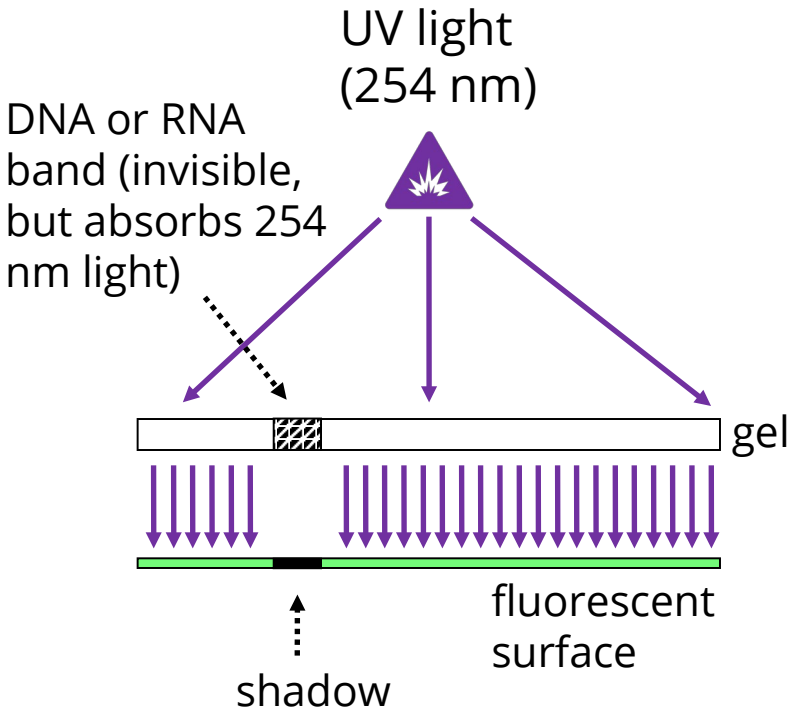
proteins get " focused" into a thin band (isotachophoresis)

Separating gel

At *high pH*, glycine deprotonates, runs with the Cl^- at the leading edge, and the proteins separate based on size

UV shadowing:
Detection of nucleic acids directly (no dye required)

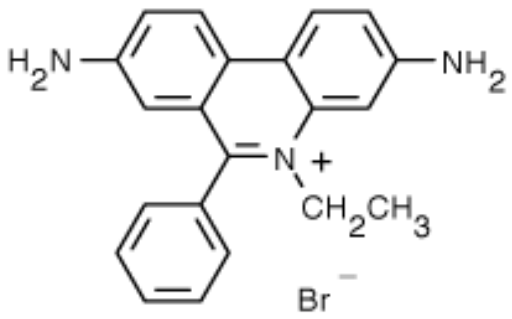




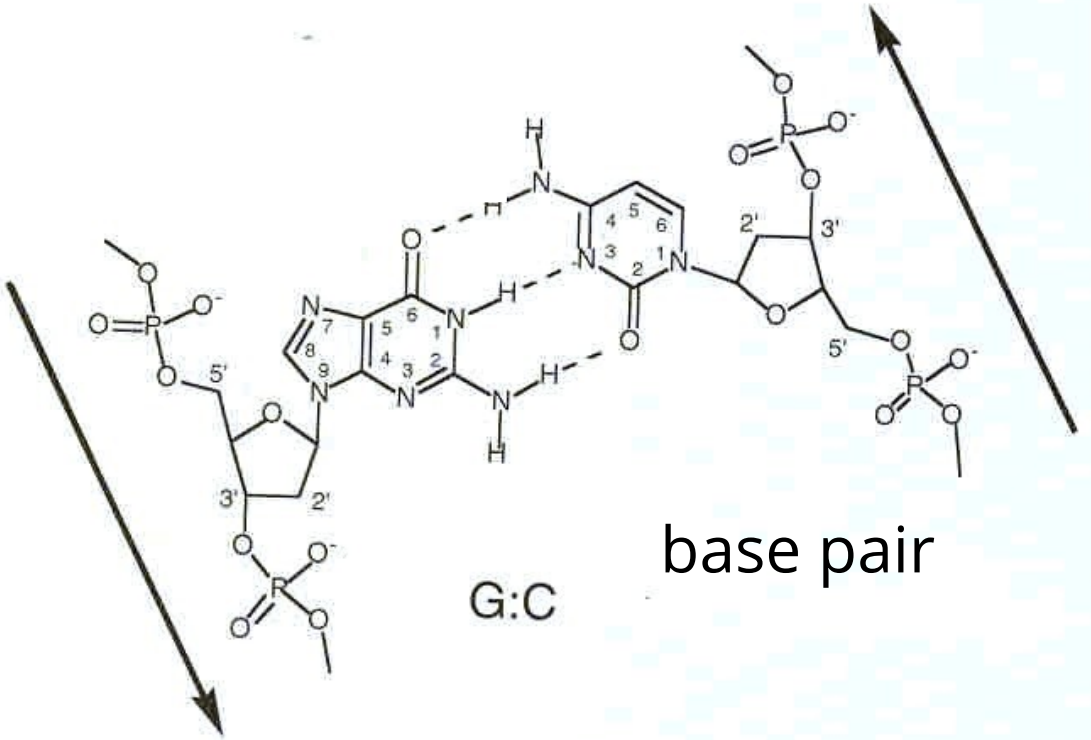
https://www.researchgate.net/figure/A-Suzuki-reaction-on-iodo-labeled-RNA-ON-4-using-1-equivalent-of-Pd-catalyst-and-100_fig6_323830929

-

Making nucleic acids visible: stains



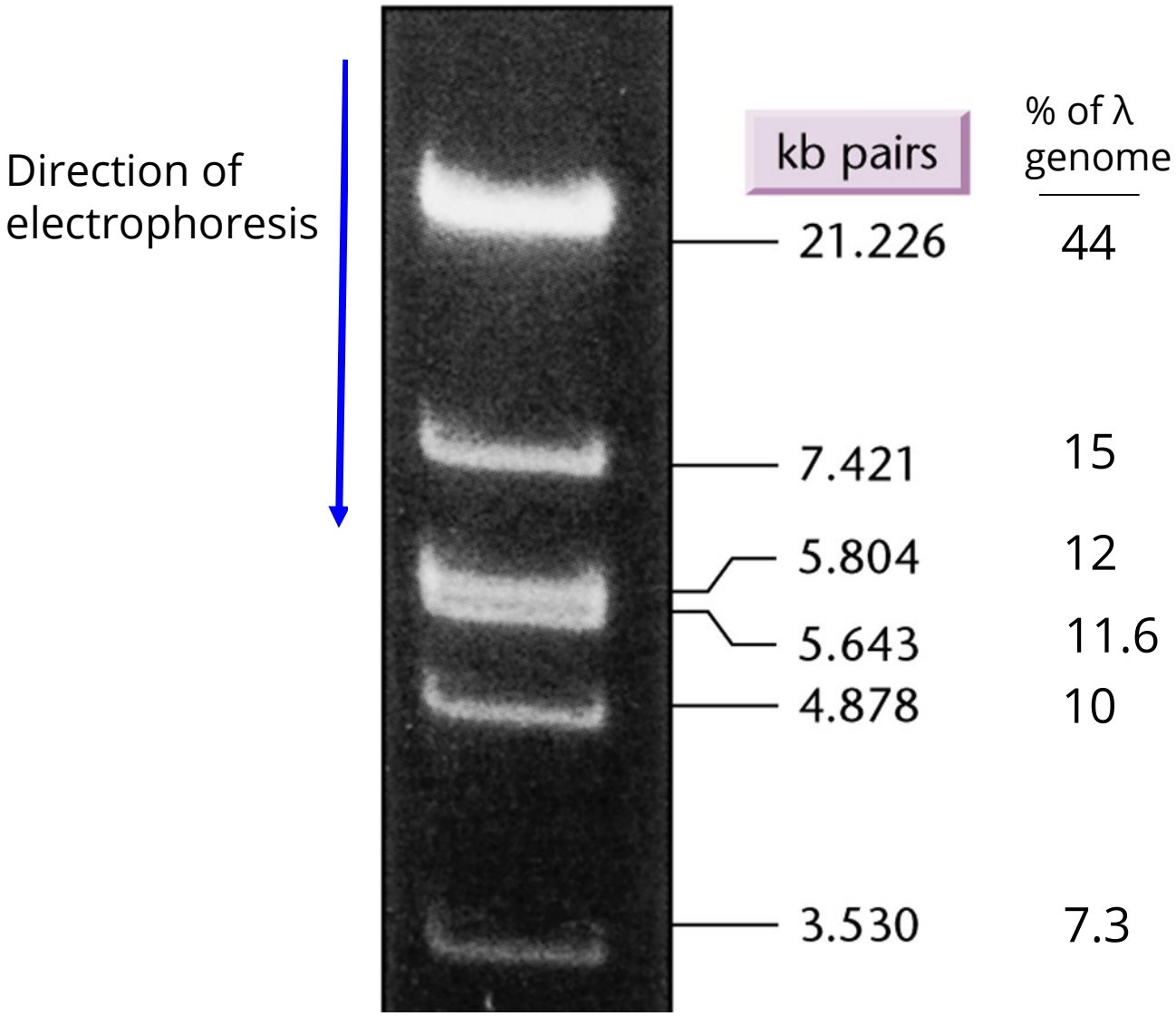
Ethidium bromide



base pair

- Ethidium bromide (EtBr) is fluorescent
- EtBr ‘intercalates’ into stacked base pairs
- Fluorescence increases upon DNA binding
- UV illumination reveals where the DNA is

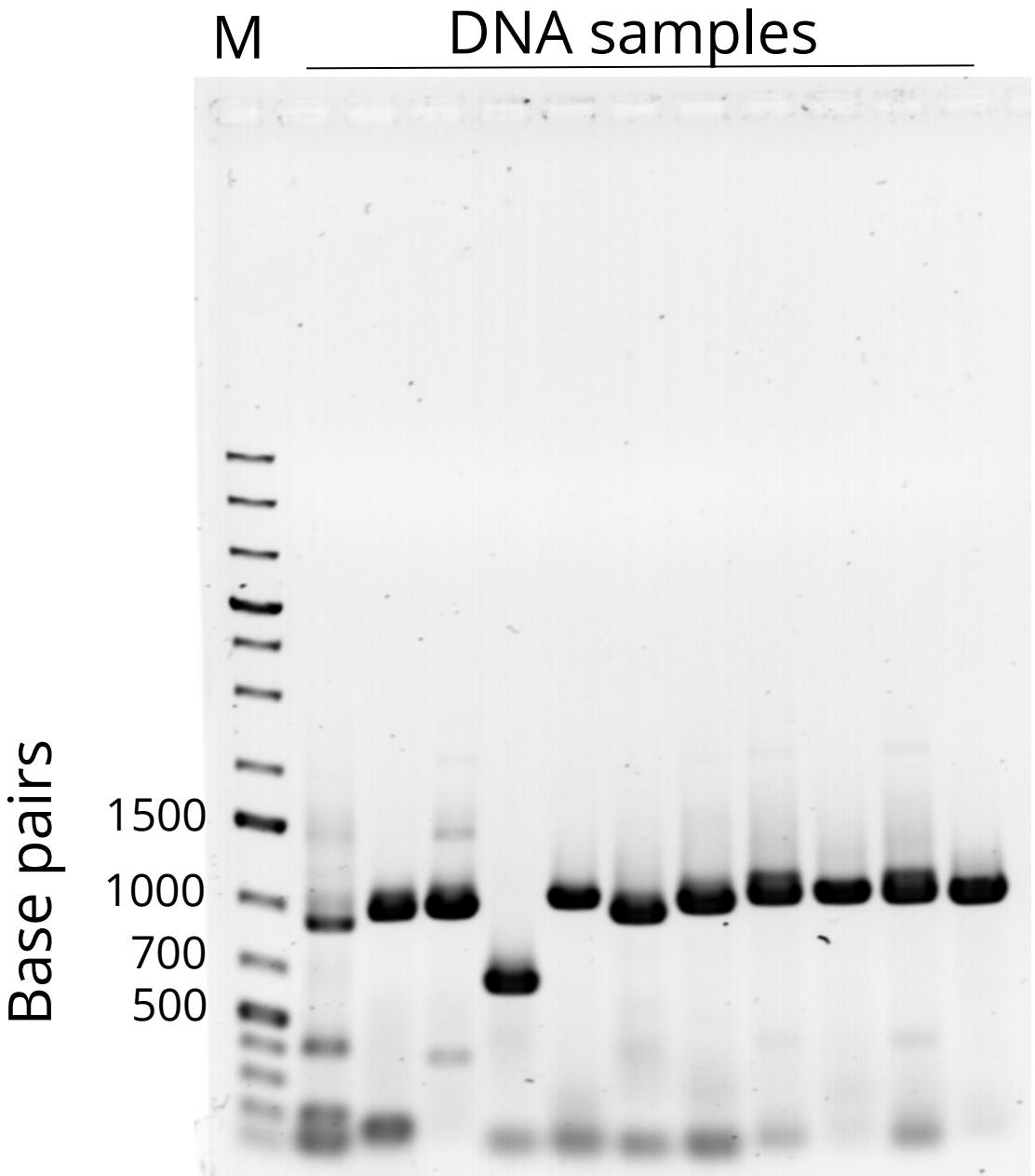
Agarose gel
stained with ethidium bromide



phage λ genomic DNA (48 kb) cut with the restriction enzyme Hind III

The fragments are equimolar--why is the band intensity different?

Marker DNA gives an estimate of size for samples



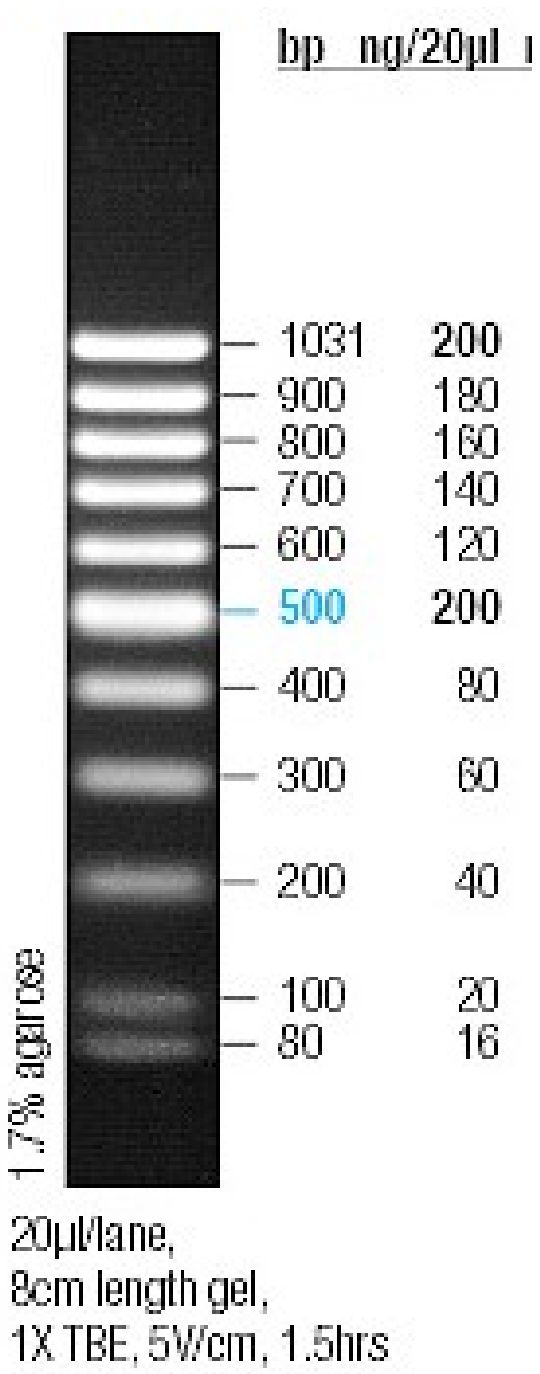
The marker lane (M) gives size standards for comparison with the sample lanes

The image was inverted to give black bands on a white background

Another way to quantify DNA:

Ethidium bromide (fluorescent dye) binding

- Compare sample DNA fluorescence to standards of know concentration (dilution series)
- In solution *or* using gel electrophoresis



A commercially available quantitative DNA standard

Other DNA staining options

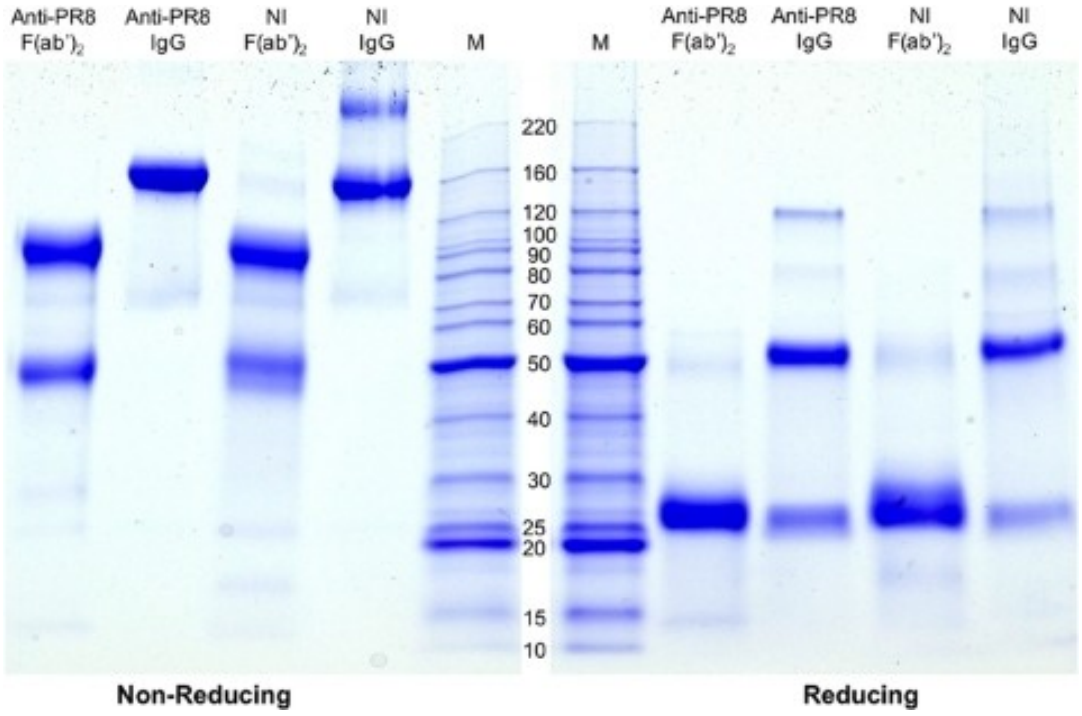
- methylene blue: staining protocol is time consuming, sensitivity is lower

- SYBR gold and other commercial options: can be more sensitive than ethidium bromide for detecting DNA, but costly

Protein detection in gels

Coomassie Brilliant Blue R-250: dye from the textile industry that has a high affinity for proteins

- Proteins in gels are " fixed" (rendered insoluble) with acetic acid/methanol
- Dye probably interacts with NH_3- groups of the proteins, as well as via van der Waals forces

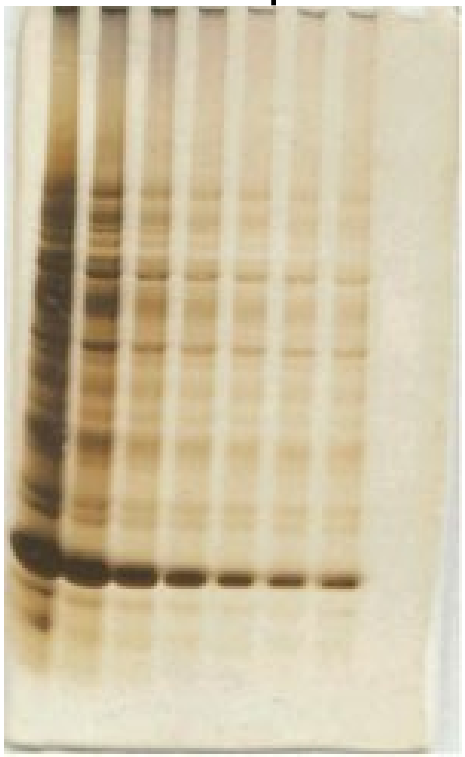


http://openi.nlm.nih.gov/imgs/512/132/2964324/2964324_pone.0013622.g001.png

Protein detection in gels

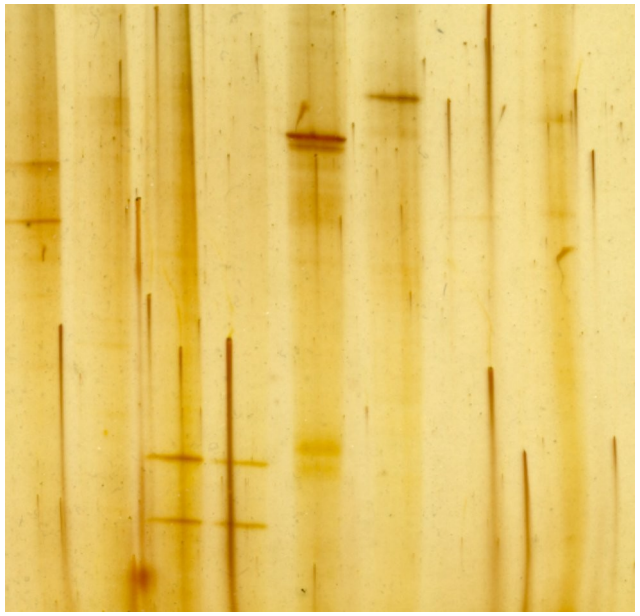
Silver stain:

- 100 to 1000-fold more sensitive than Coomassie (requires far less sample)
- Silver in solution interacts with amino acid side chains and is selectively reduced (similar to early photographic process)
- There is protein-to-protein variability of staining



good

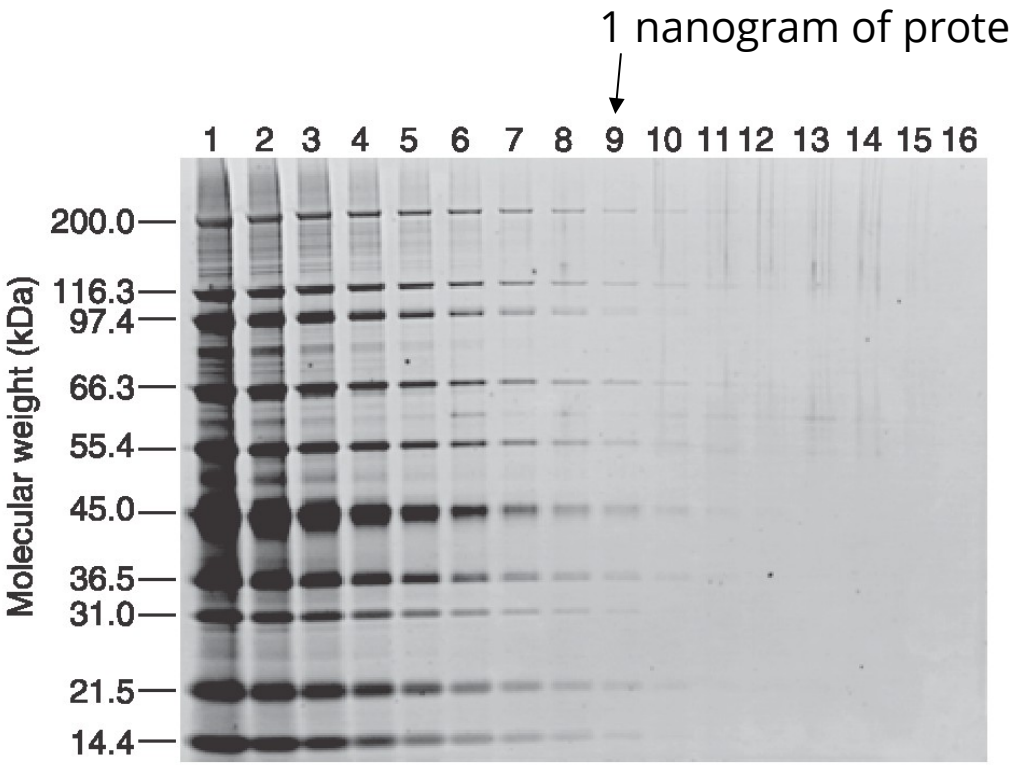
bad (overstained)



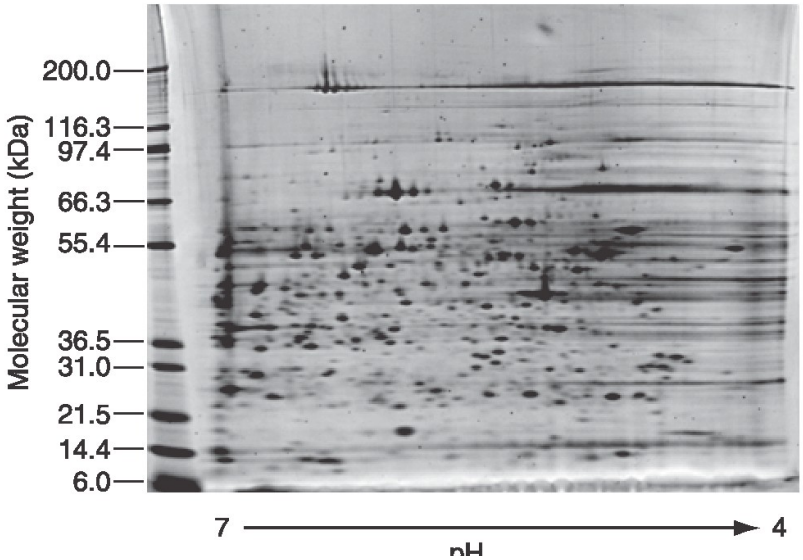
ugly (keratin)

Protein detection in gels

- Sypro Ruby (Molecular Probes inc, proprietary compound)
  - As sensitive as silver staining, less variability
  - Fast protocol
  - $



Standard gel



2-D gel

Visualizing DNA (and RNA, protein): non-specific detection methods

I. Quantitation of nucleic acids (chemical properties: bases, dye binding)

II. Electrophoresis (PO_4^- groups, size)

III. Visualizing macromolecules (dye binding)

Note:

Many protocols can be found at http://openwetware.org