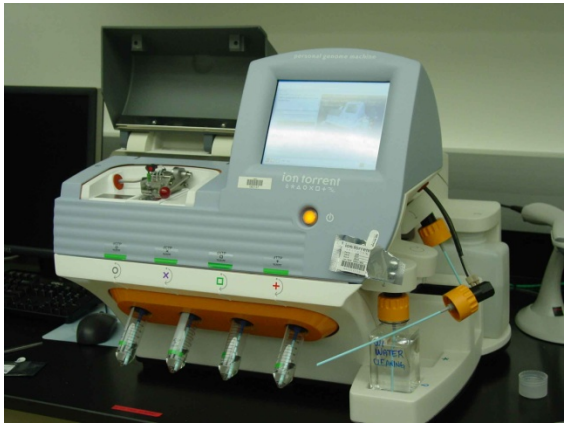
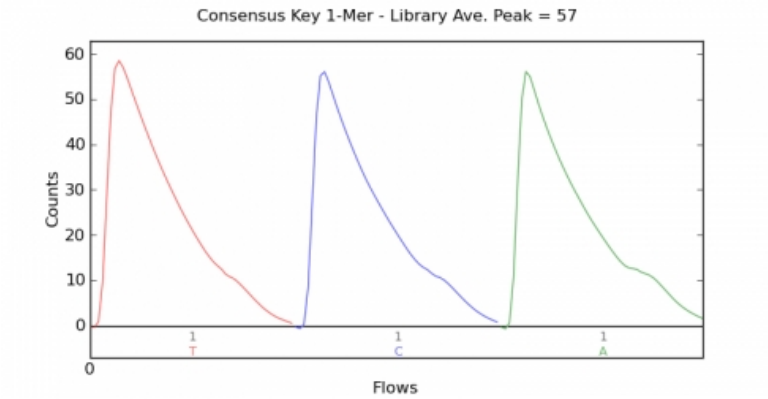
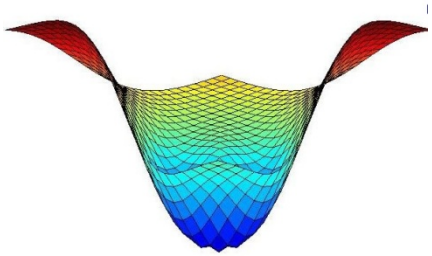
BINF 6010 ITSC 8010
Fall 2011
Genomic Biotechnology Lab
Spectrophotometry and Electrophoresis

http://webpages.uncc.edu/~jweller2

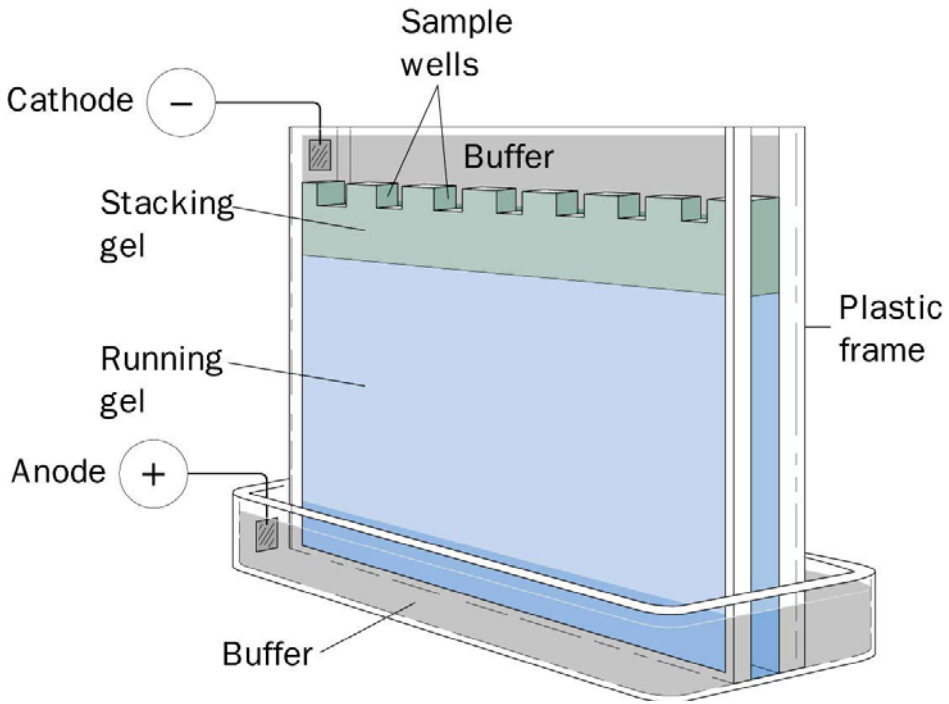




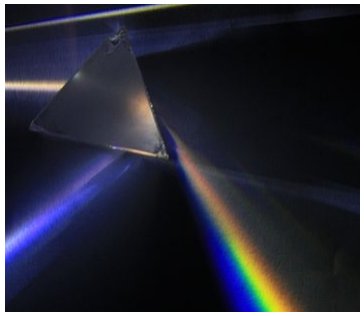


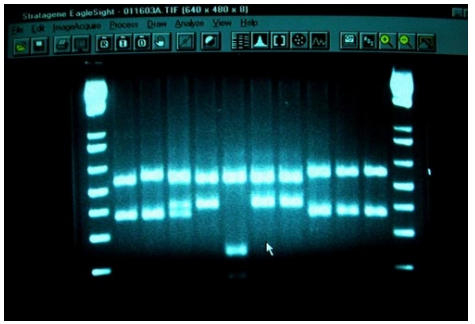
Topics

- Spectrophotometry
- Electrophoresis

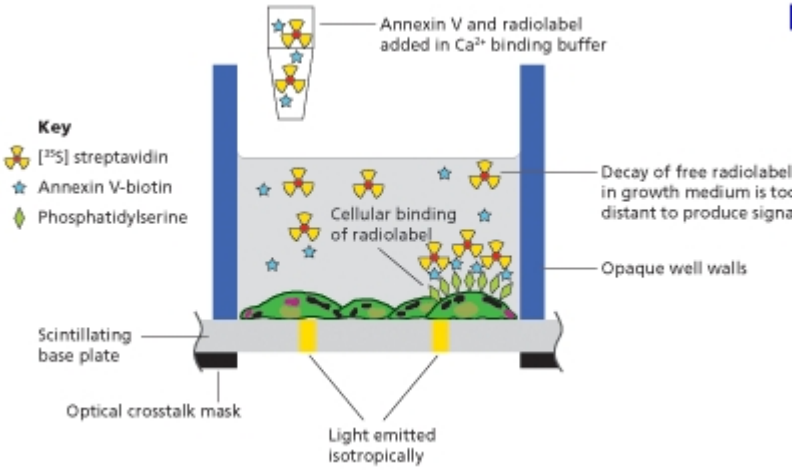


Sample preparation is monitored at each handling stage by expected yield and integrity of the molecule(s).



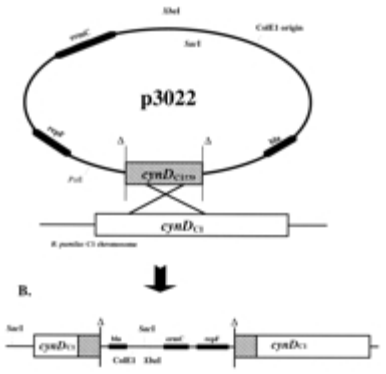


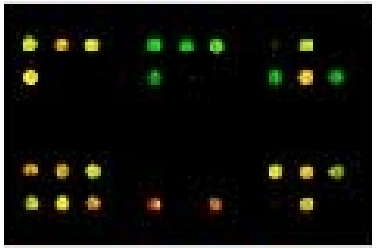
- Identity
  - Direct assays
    - a physical property like mass, interaction with light of specific frequencies, length
  - Indirect Assays
    - Add a group and measure that group (e.g. radioactive labeling)
- Purity
  - Sensitivity of assays
  - Specificity of assays



Assays can be characterized as open or closed depending on whether unexpected events can be observed.

- Open Assays are able to show something has occurred (although your assumptions may limit your interpretation).
  - If a plasmid that is normally 20kb loses a large amount of its material and is only 5kb in length, a gel will show the loss of one product and gain of another.
- Closed assays restrict the type of information that is acquired – they are simpler to interpret but major events may be missed entirely.
  - If the test of a plasmid's presence is for a short region of the DNA, and that DNA is part of what is lost in the rearrangement then the test will indicate loss of the plasmid rather than a change in its form.





Method 1: Spectroscopy measures how matter interacts with radiation.

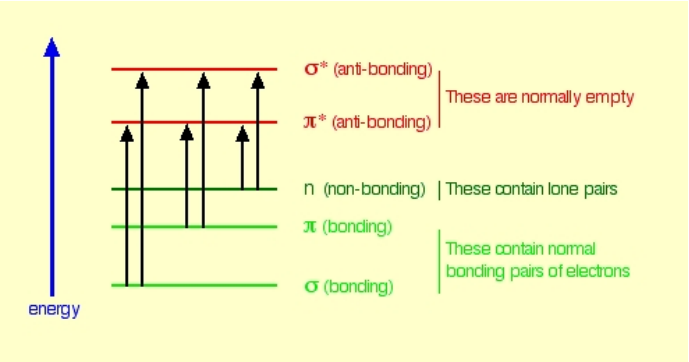
$$E = h\nu$$

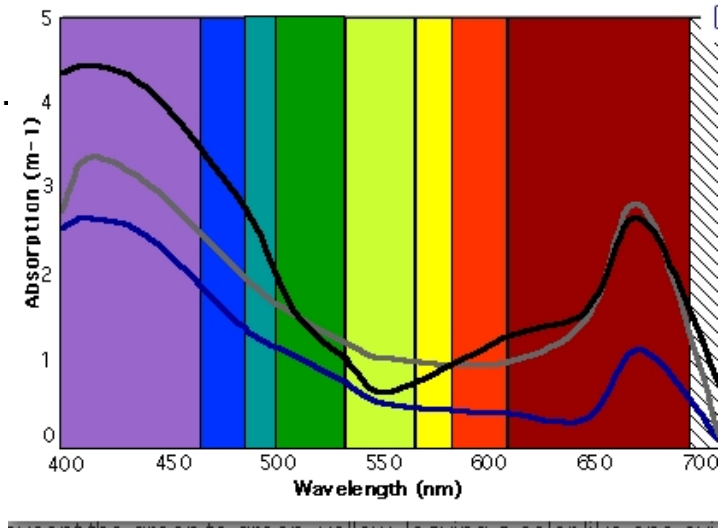
$$\lambda = \frac{c}{\nu}$$

- Radiation is characterized by frequency or wavelength.
  - Radiation passes through or bounces off matter (Δ intensity, frequency shift, etc)
- Absorbance
  - Interactions are frequency dependent so monochromatic light is used.
- Scattering
  - Photons reflect from the material – coherent light will change direction
- Fluorescence
  - Interactions with molecules causes increases in electron orbitals with energy lost as photons as they decay

When light is absorbed by molecules the energy of the photon may promote an electron to a new orbital.

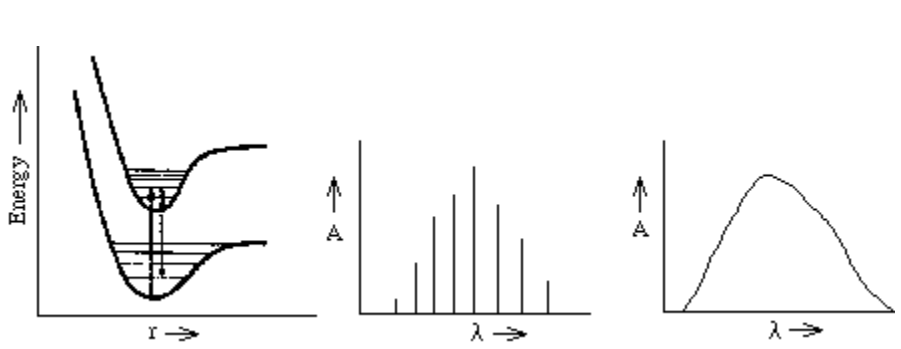
- The energy provided has to match the energy difference in the orbitals, changing the state of the molecule.
- This energy can be released by electron decay to the original state or a new state, with photon release, or by transfer to another molecule or dissipation to the environment.
- For proteins and nucleic acids, photons in the UV and near-UV-visible range have the right energy to be absorbed.
- Organic molecules subjected to UV-vis light have transitions from
  - pi bonding to pi antibonding orbitals
  - non-bonding to pi antibonding orbitals
  - non-bonding to sigma anti-bonding orbitals.

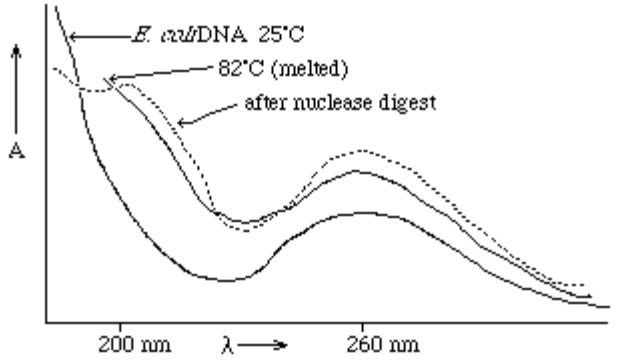




Absorbance spectrum

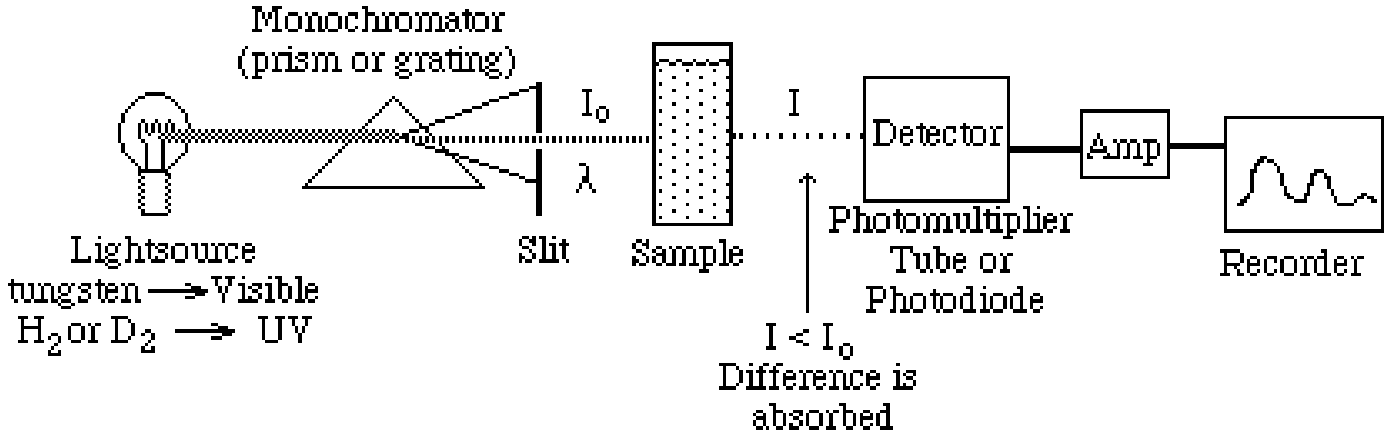
- For light passing through a solution of identical molecules the rate at which photons are absorbed is a function of the distance (length) through the sample holder (a cuvette), proportional to the concentration (c), at a rate having a constant called epsilon (ε).
  - Biopolymers have many vibrational energy levels so there are many closely-spaced absorption peaks that sum to a broad peak.





Measuring the changes in light as it interacts with matter is called spectrophotometry

- A spectrophotometer selects light of a determined wavelength from a source, passes it through a sample, and detects the number of photons (intensity) and/or frequency of the wavelength that reaches the detector.



For biomolecules the two most common types of spectroscopy experiments yield a spectrum or a concentration.

- A spectrum is a kind of fingerprint, - determine across a range of wavelengths which ones a sample absorbs and transmits
- At a specific wavelength determine how much of a molecule is in a solution.
  - Use Beer's Law (or the Beer-Lambert variant) to calculate the concentration
    - $A_\lambda = \varepsilon cl$

    Where the Absorbance (at a given wavelength) equals the extinction coefficient, ε (mass or molar) times concentration (units better match ε) times the path length (standardized to 1cm).

For mass and nucleic acids that are collections, the ε = 50 ml/ng-cm for dsDNA, 40 for RNA and 33 for ss DNA.

Dye Type	Extinction Coefficient (liter/mol-cm)	Measurement Wavelength (nm)
Cy3	150000	550
Cy5	250000	650
Alexa Fluor 488	71000	495
Alexa Fluor 546	104000	556
Alexa Fluor 555	150000	555
Alexa Fluor 594	73000	590
Alexa Fluor 647	239000	650
Alexa Fluor 660	132000	663
Cy3.5	150000	581
Cy5.5	250000	675

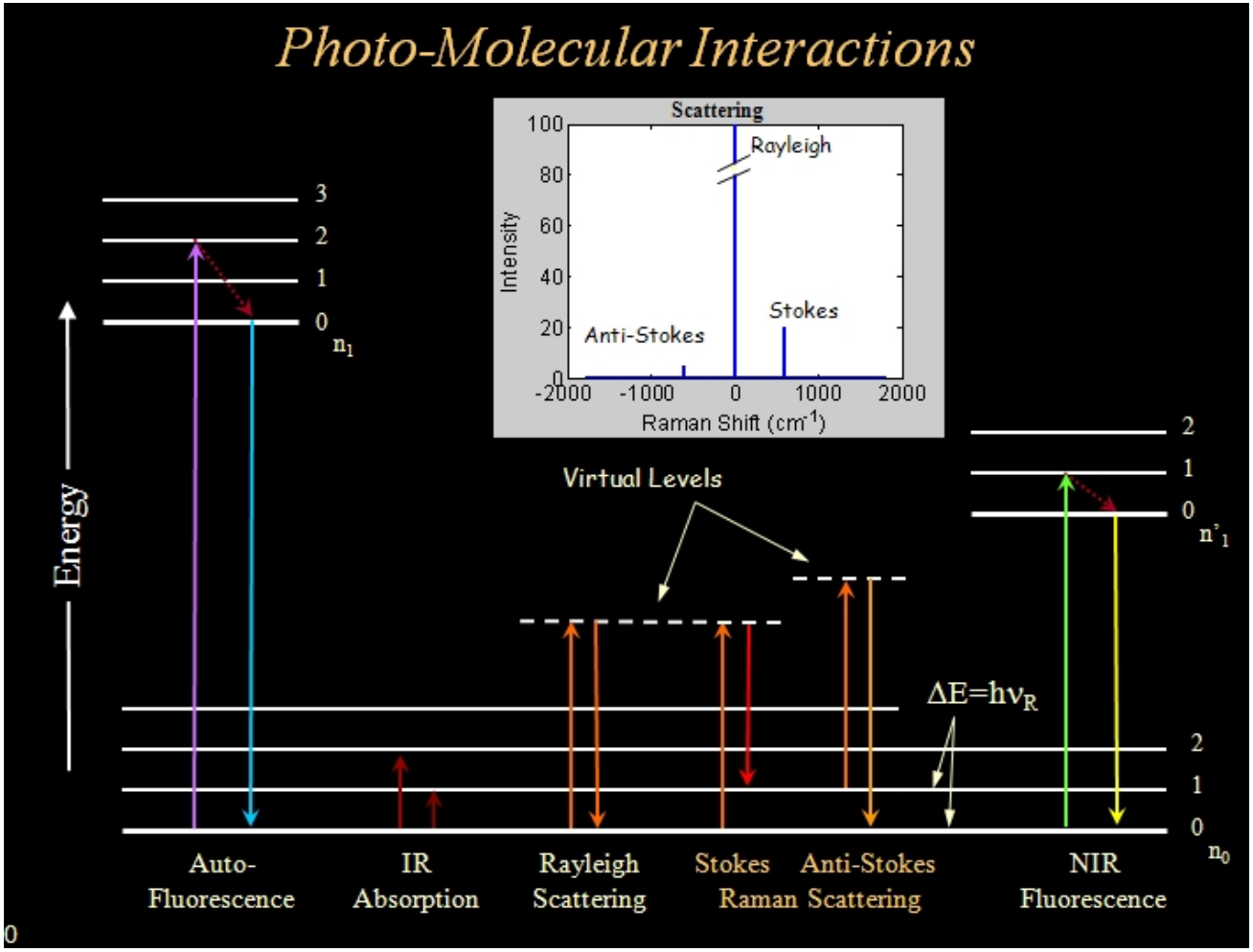
Simulations using spectrophotometers

- A virtual lab showing concepts important to spectrophotometry, including taking an absorbance spectrum, or light-responsive fingerprint of a molecule across a group of wavelengths: http://www.chm.davidson.edu/vce/spectrophotometry/
- Although we will be using different platforms, there is an animation of common experimental steps here: http://ncbionetwork.org/spectrophotometer/
- A video (in two parts) of a biochemistry lab measuring Riboflavin http://carrollslab.blogspot.com/2008/08/biochem-lab-spectrophotometry-of.html

There are additives one can use to increase the sensitivity or specificity of detecting a type of molecule – specifically dye-modified reagents.

- Specificity enhancement – a reagent that interacts with one component of the target, or one component in one conformation. Dyes that
  - interact specifically with single or double-stranded nucleic acid
  - specifically with either DNA or RNA
  - Recognize one base, one modified base, or a short sequence of bases
- Sensitivity enhancement
  - Use dyes with a high quantum yield, or having an associated amplification reaction (e.g tyramide).
    - Minimize the NA contribution at the emitted wavelength.

- For polarizable molecules an incident photon can excite vibrational or rotational modes.
- Rayleigh scattering is elastic – the energy lost is of the same frequency as that absorbed – vibrational modes are involved.
- Raman scattering is when the energy released is greater or less than the incident photon energy (Stokes and anti-stokes) - lots of imaging applications.

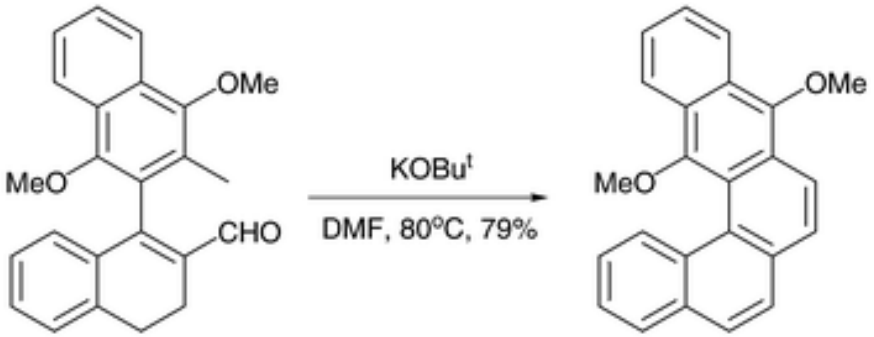


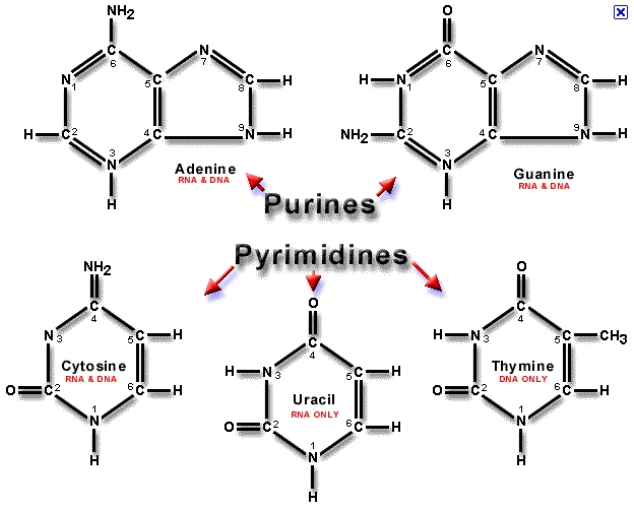
Fluorometry

- Spectrophotometry is a direct assay – a characteristic of the molecules is measured directly.
  - Not always sufficiently sensitive.
- A fluorometer allows indirect assays with engineered dyes having better characterisitics
  - Ideal: high affinity and binding constant, photons emitted only when bound to MOI, simple binding mechanism and binding ratio.
- For very small amounts of DNA a fluorometer gives a more sensitive reading of the concentration.
  - The units are relative rather than absolute (efficiency of attachment as well as interaction of dye and polymer have to be considered – may be buffer and pH dependent).
    - Thus, after zeroing with a “blank”, always begin an assay by calibrating the instrument to display the *known concentration of a standard solution.*

Fluorescence is the result of a 3-stage process occurring in molecules with certain characteristics

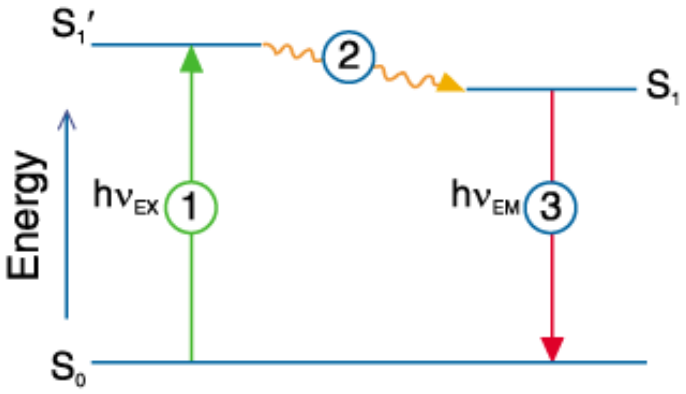
- Polyaromatic hydrocarbons
- Heterocyclic compounds like bases

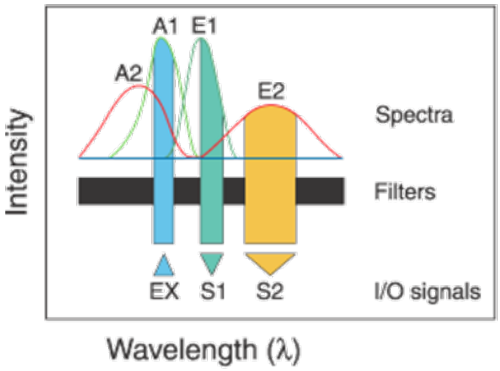




The Jablonski diagram summarizes the 3 stages of Fluorescence.

- Stage 1: excitation, where a photon (energy $h\nu_{EX}$) is supplied (from incident light or an energy source)
  - The fluorophore absorbs the photon, an electron is raised to a singlet state (S_1')
- Stage 2: S_1' is short-lived (1-10 nsec), the electron decays to S_1, dissipating energy.
  - The fluorophore undergoes conformation changes and interacts with the environment – a photon may be released or the energy may be lost.
- From the relaxed singlet state S_1 the electron drops back to S_0, the stable ground state, releasing the energy as a photon
  - The energy of the photon is less, so the wavelength of emitted light is longer. The difference between the excited and emitted wavelengths is called the Stokes shift

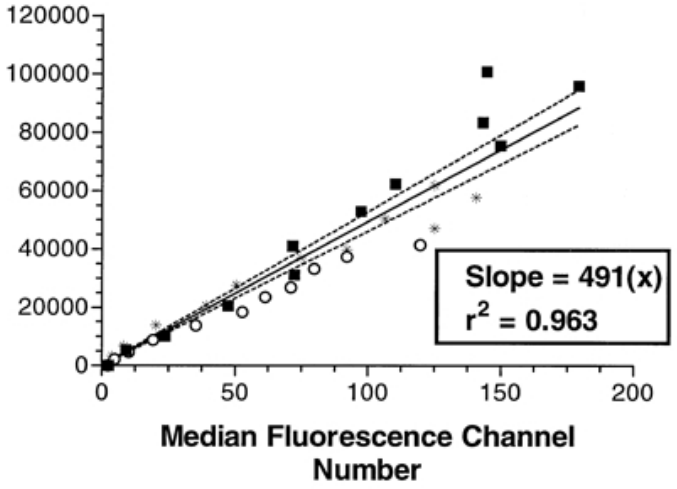




Fluorescence is an indirect assay for detection is more complicated than simple UV absorbance.

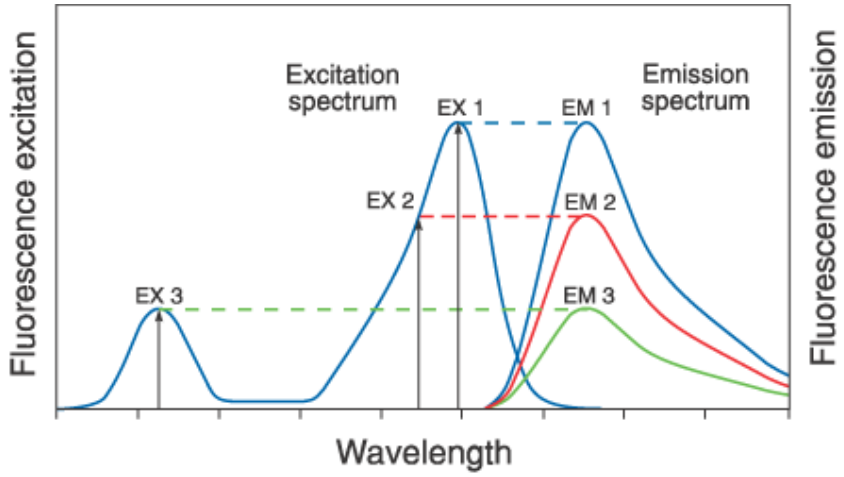
- Instruments have the same set of requirements as absorbance spectrometers – a responsive molecule (fluorophore, with high qunatum yield, little photo bleaching and little dark release) must be part of the system.
- Fluorescent intensity also obeys the Beer-Lambert law $I = \varepsilon cl$
  - The extinction coefficient has to factor in:
    - The quantum yield (QY) of the fluorophore is the ratio of the number of photons emitted in Stage 3 to the number effectively absorbed in Stage 1.
    - The intensity of the excitation source
    - The collection efficiency of the detector – thus the need for a calibration standard

The emission intensity is linearly proportional to the amount of dye when the concentration is low (dilute solution; the absorbance should be <0.05)



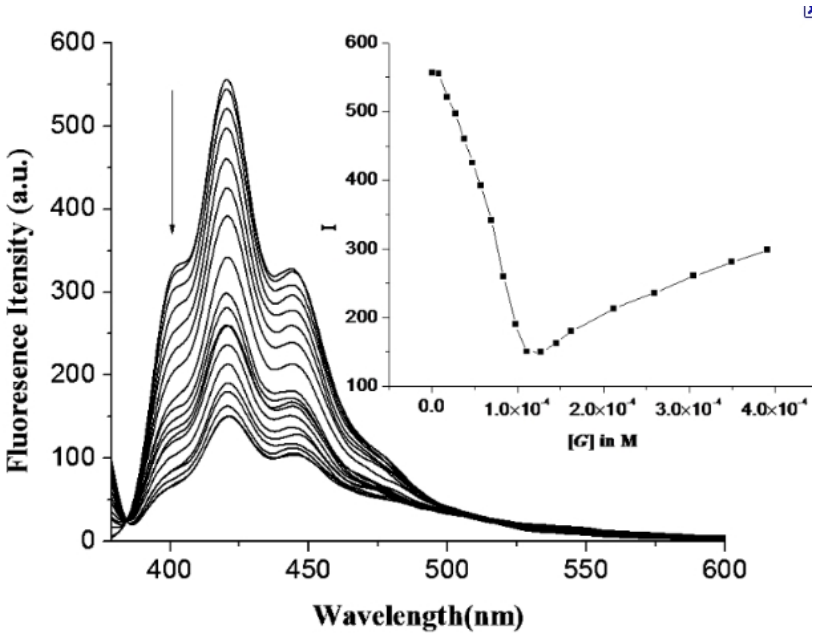
Fluorescence Spectra

- The excitation, decay and emission process occurs in repeating cycles → repeated signal from the same original source is why sensitivity is enhanced.
- The excitation band (for a single molecule in dilute solution) is identical to the absorption spectrum
  - The breadth of the emission bands is important when choosing multiple dyes, so there is not too much overlap.



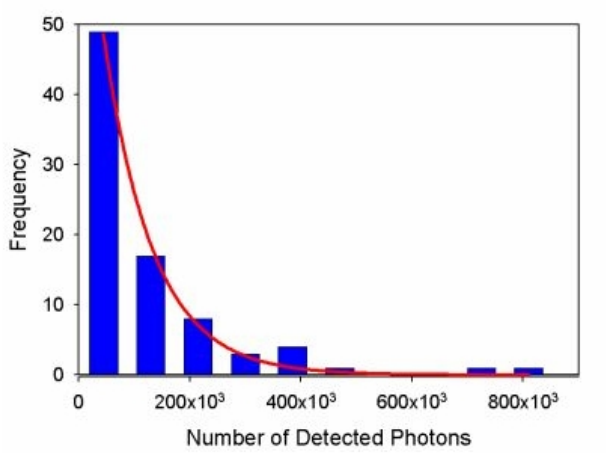
If there are so many dependencies, how reliable is this method?

- When solvent and temperature and concentration stay the same
  - the position and shape of the emission spectrum is *independent* of the excitation spectrum
  - The intensity (amplitude) of the emission is proportional to the amplitude of the excitation intensity at the maximum.



Factors that can influence the reproducibility of the fluorescence readings include contaminants and byproducts.

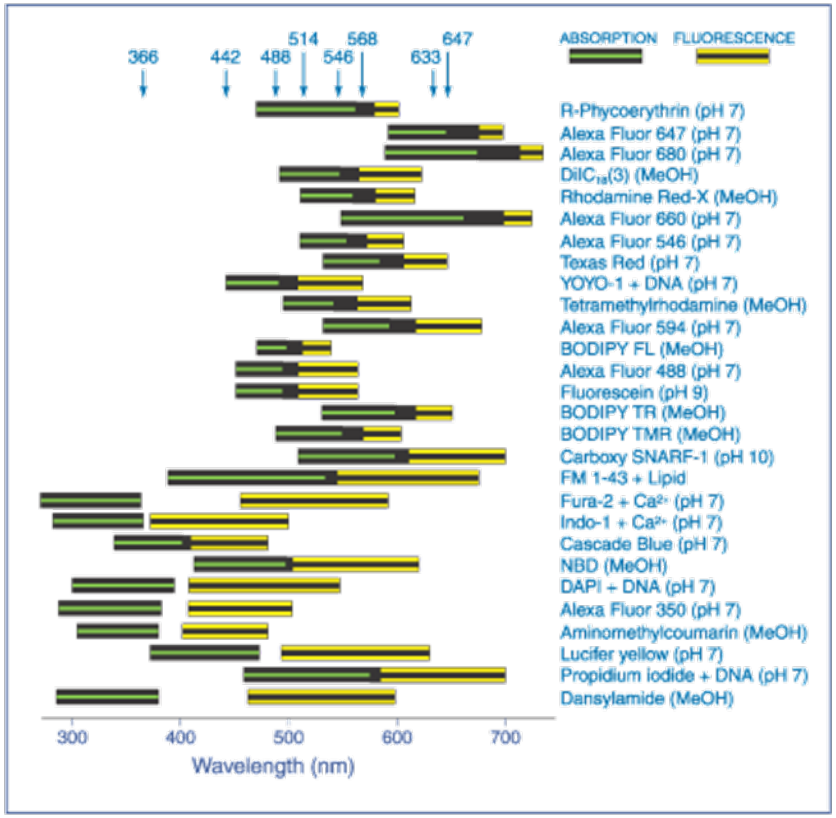
- Background sources
  - Autofluorescence from the sample (eg the bases in DNA)
  - Unbound or non-specifically bound dye
- Photobleaching (destruction of the molecule form the light)
    - Starts at the triplet excited state: inter-system crossing occurs from S_1



The Molecular Probes Handbook is a great source for explanations and choices when selecting Fluorophores

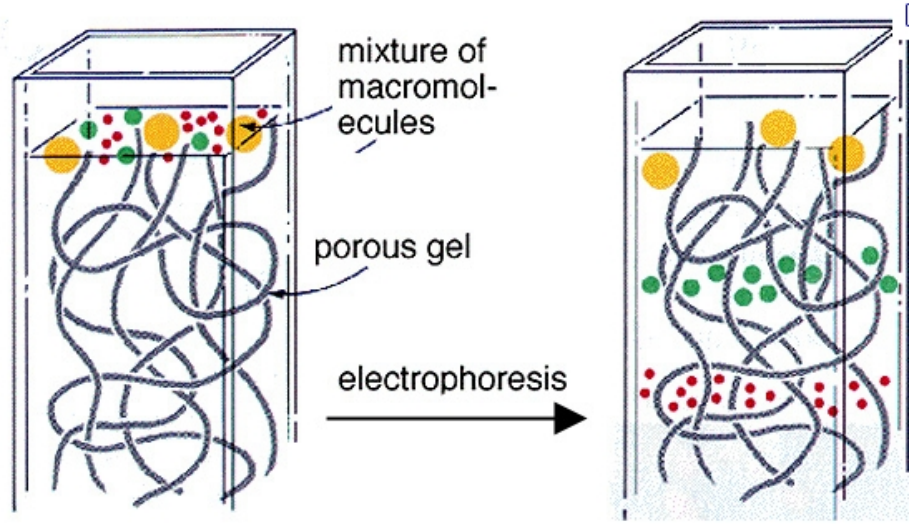
- The instrument and attachment chemistry constrain the choice of dyes.

- Measures to consider include
  - Fluorescent output: the efficiency of absorption and emission and the cycling rate
  - The valid conditions (solvent, temperature and wavelength)
    - The molar extinction coefficient of absorption
    - The quantum yield
  - The I_{fl} (per molecule) α [ε*QY]
    - Ranges are 5000-200,000 $cm^{-1}M^{-1}$ for ε
    - 0.05-1.0 A_{hv} for QY



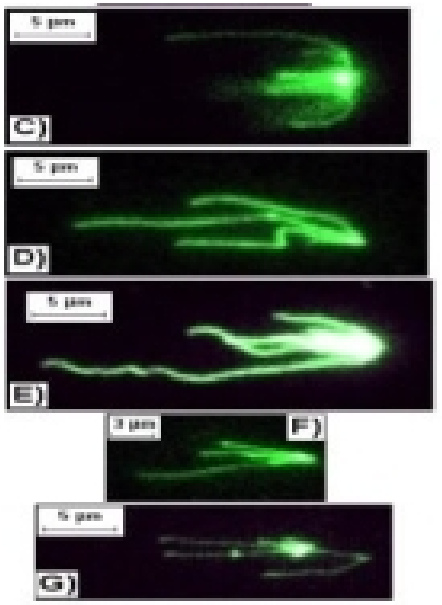
Method 2: Gel electrophoresis is the separation of molecules in a charged field using a sieving matrix.

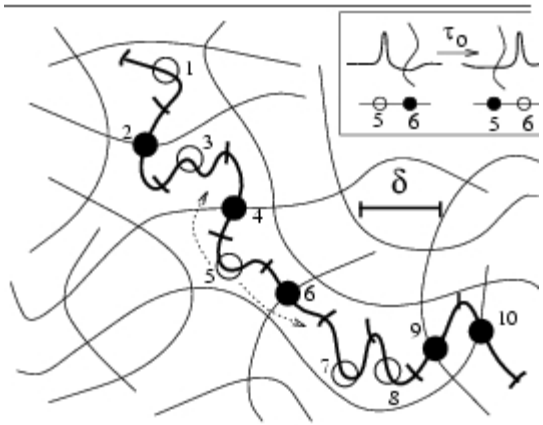
- Charged molecules will migrate under a voltage potential.
  - The environment and the total charge density (charge:mass ratio) and shape affect the rate of migration
- A gel matrix is mostly liquid but does give some inert support – there is an average pore size of interlaced strands.
  - The matrix retards diffusion, convection, etc.
  - The pores impose some size/shape selection



Nucleic acids have a constant charge:mass ratio from the phosphate backbone.

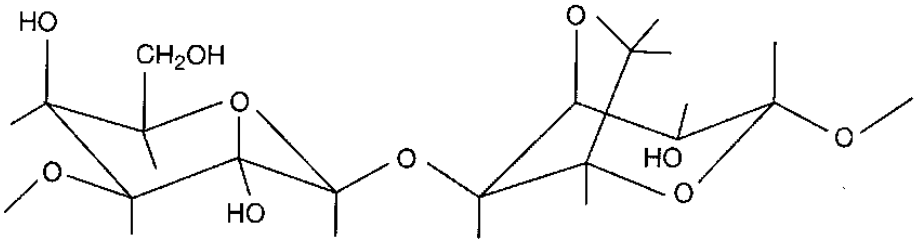
- Duplex NA are rather stiff and rod-like.
  - In solution they migrate at the same rate (barring frictional effects).
- In a gel the pores slow down longer molecules more than shorter ones (jointed segments).
  - The gel is ‘cast’ as a thin slab (vertical or horizontal), with wells on one side to hold the sample.
  - The edges of the gel are immersed in electrophoresis buffer so that there are ions to carry the current and to maintain the pH.





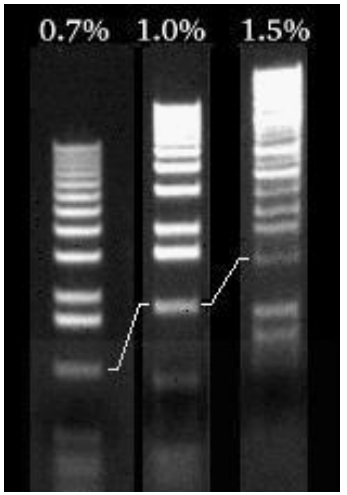
An agarose gel is made of long polysaccharides extracted from seaweed.

- The polysaccaharide is used at 0.5-3% concentration (wt/vol).
  - Higher conc → smaller pore size
- The range of separation is quite large (100-50,000 bp) but the resolution is rather low.

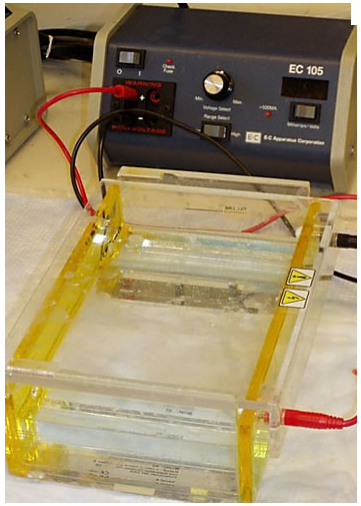


D-galactose

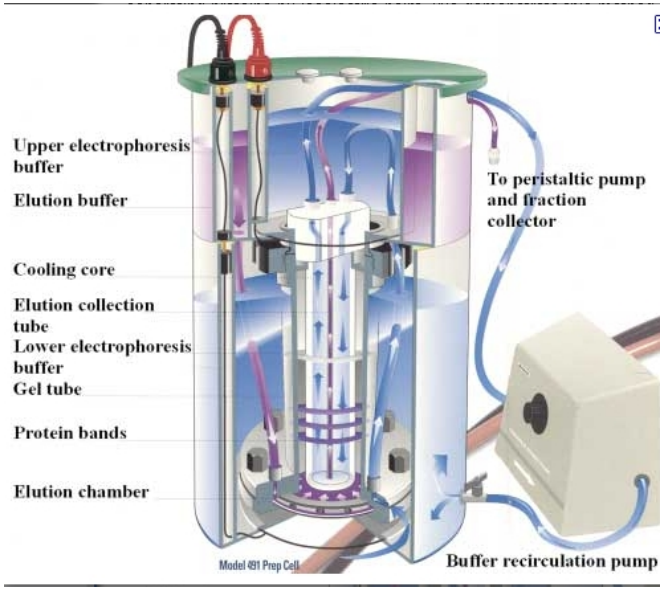
3,6-anhydro L-galactose

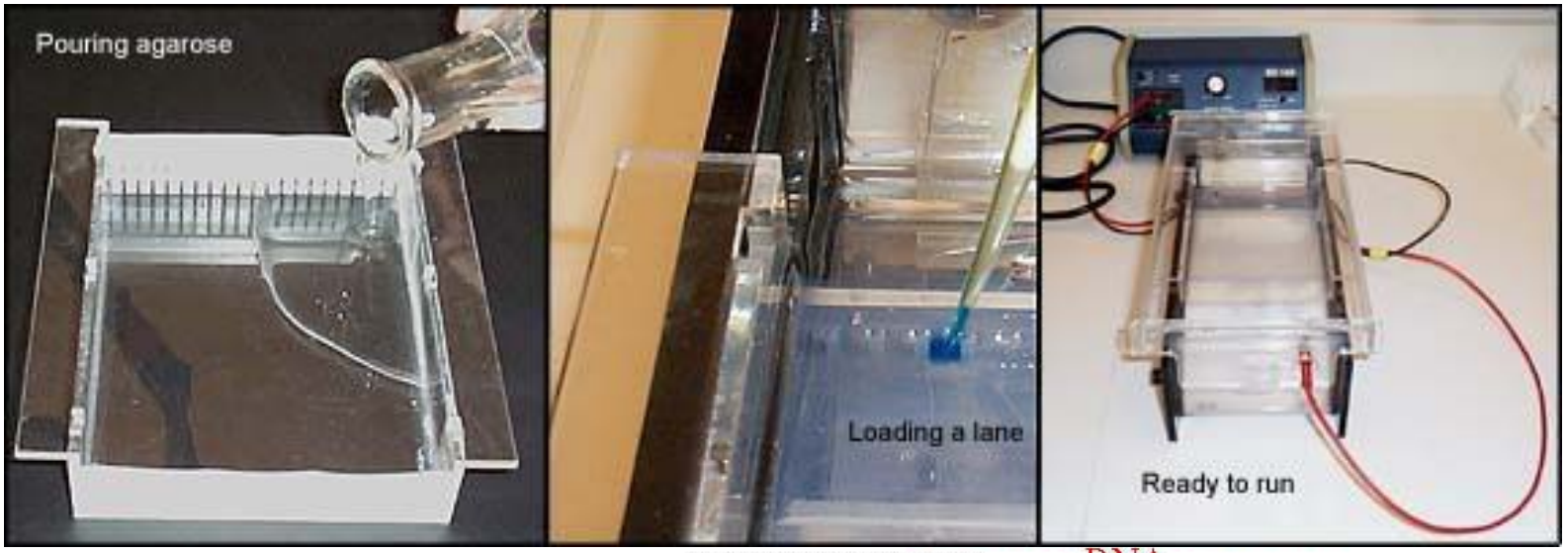


Instrument components are shown on the next page, named here.



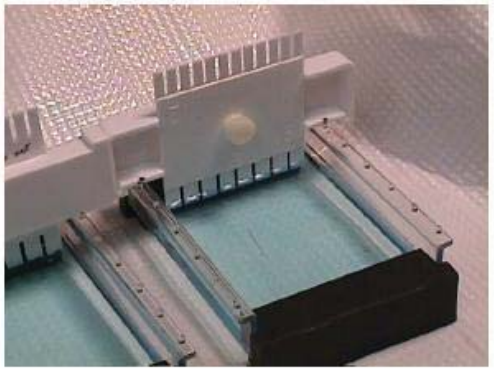
- A power supply, leads, electrophoresis chamber with buffer reservoirs, gel cassette.
- Power supply maintains constant voltage or power
  - the velocity that the sample moves is proportional to the voltage; resistance changes over time.
  - As the gel length increases the resistance increases by $R_g(I_2/I_1)$.
  - As the gel percentage (D) increases the resistance decreases by $R_g(D_2/D_1)$.
    - A cooling fan may be used if the resistance is large
    - The buffer may be recirculated if the run is very long



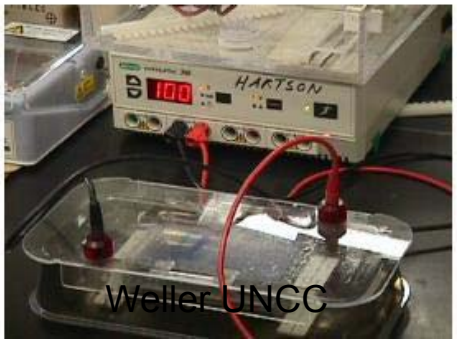


PREPARATION OF A GEL

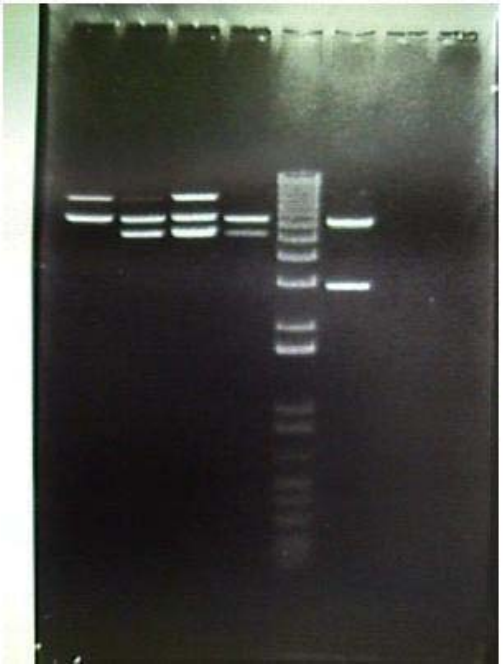
DNA



RUNNING IN AN ELECTRIC FIELD



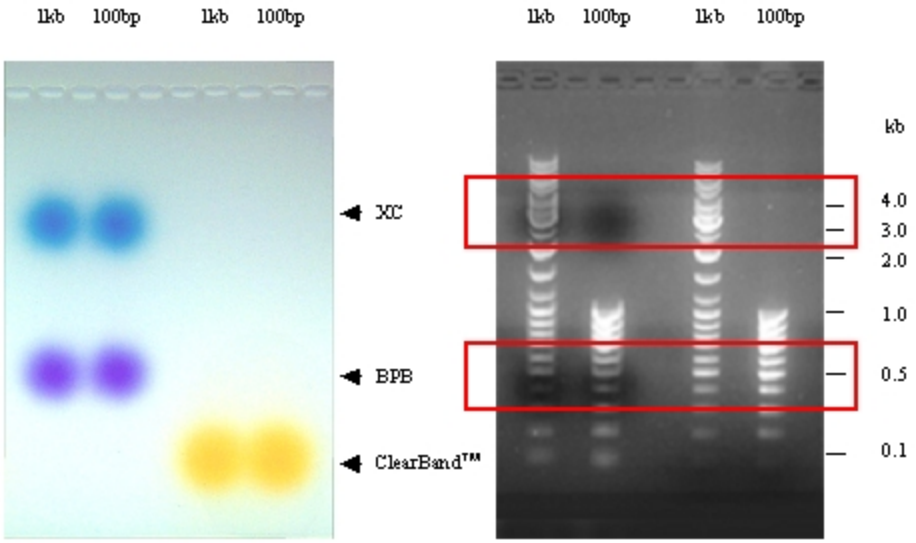
STAINING AND DETECTING BANDS OF DNA FRAGMENTS



A number of commercial suppliers also provide support for selecting the right concentration and types of agarose to use.

Recommended Agarose Percentage for Size Separation

% Agarose	Size Fragments Separated (bp)
0.5	1,000 to 30,000
0.7	800 to 12,000
1.0	500 to 10,000
1.2	400 to 7,000
1.5	200 to 3,000
3-4 (sieving agarose)	10 to 1000

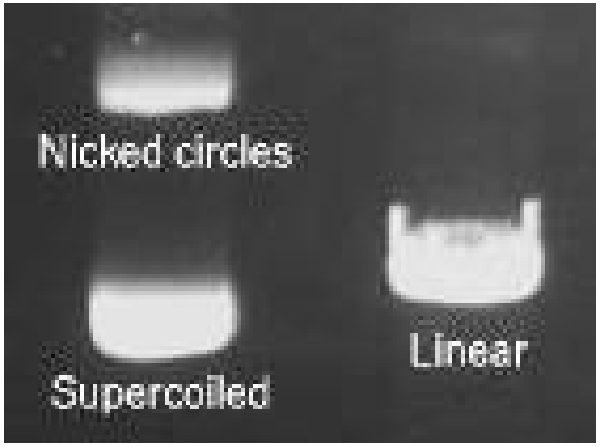


Dyes are used to track and visualize the progress of sample separation in the gel.

- Visible dye markers are used to judge approximate travelling distance of the sample in the gel.
  - Bromophenol blue and xylene cyanol are the most common tracking dyes.
- To visualize the samples, fluorescent dyes are most commonly employed.
  - Ethidium bromide is the most widely used
    - It intercalates between bases of either DNA or RNA – it can be used in the gel, or added after elecrophoresis
      - It is toxic (a carcinogen) so it must be disposed of as hazardous waste.
      - The wavelength at which is fluoresces is in the UV, so documentation requires an illumination source that emits UV and a recording source sensitive to UV.
- A You-tube video showing mini-agarose gels being poured and run: http://www.youtube.com/watch?v=QEG8dz7cbnY&feature=related

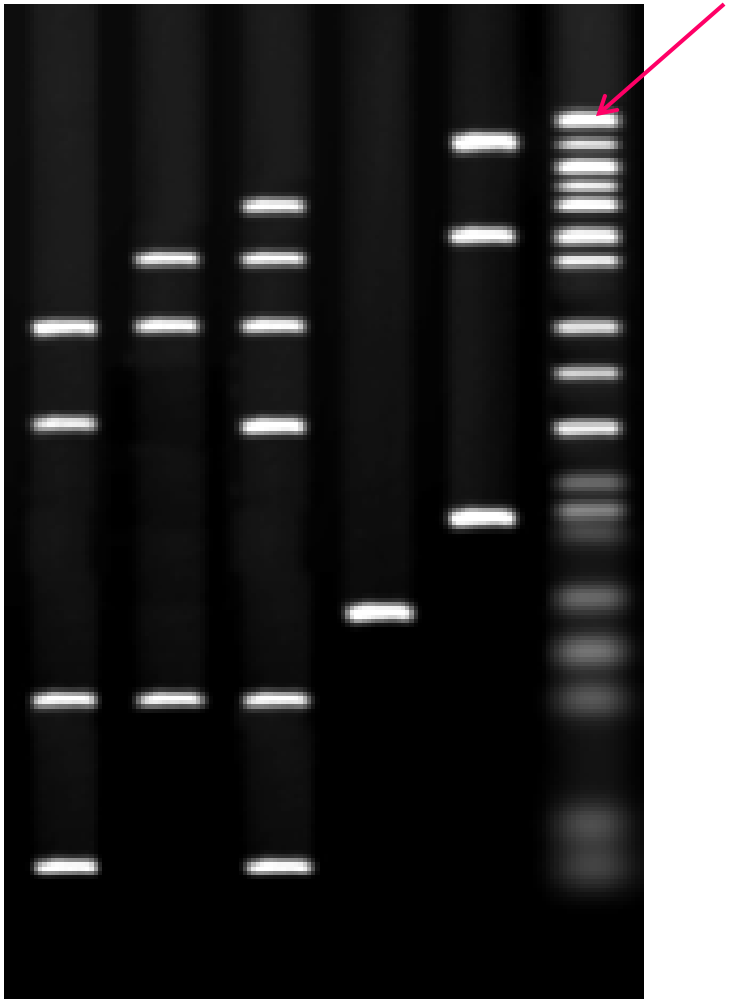
Analysis of the gel electrophoresis results has to take the stain and the state of the molecules into account.

- Ethidium bromide in binding to the DNA alters the mass and alters the rigidity - so it alters the mobility if you add it upon sample loading.
  - Can you use this? (e.g. finding conformers)
    - Circular DNA usually migrates faster
    - Linear DNA mobility α $(\log_{10}mw)^{-1}$
    - Plot using $D=a-(b*\log_{10}MW)$



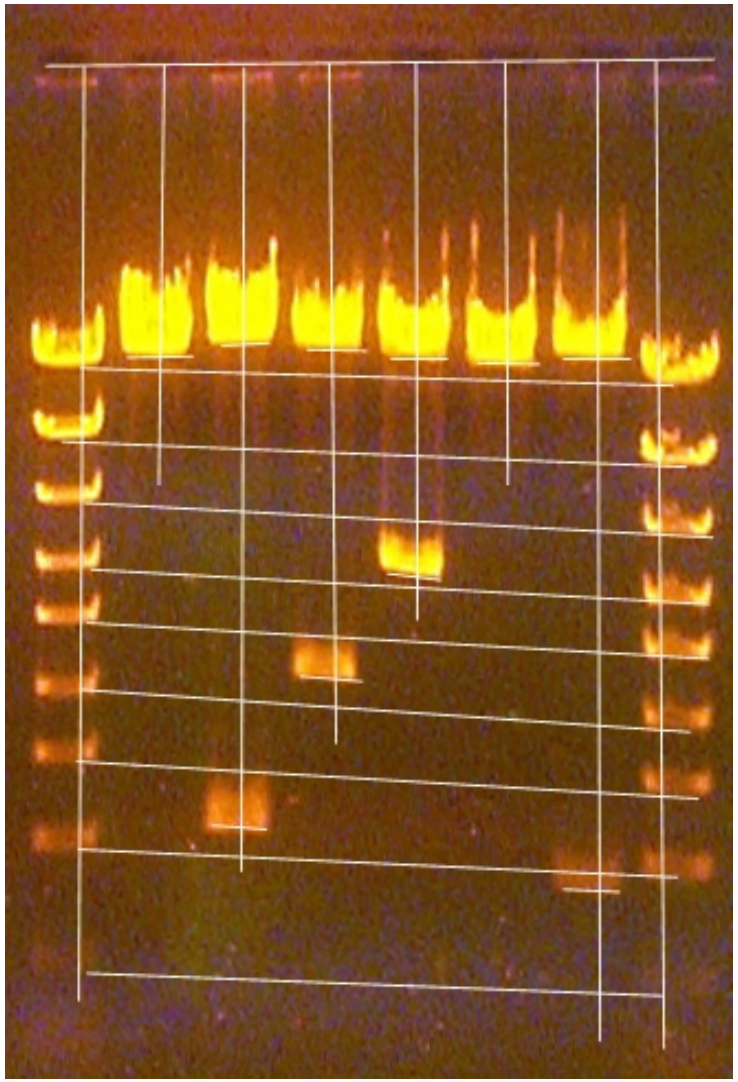
Calculating gel parameters requires the inclusion of size standards and use of a sensitive dye

- EtBr: UVmax at 300 and 360nm, absorbs energy from nt at 260nm and re-emits at 590nm. Background when not intercalated is low.
- Detection limit is 1-5ng/band in agarose.
- If staining ssNA, you need 10X more material.
- Polyacrylamide quenches EtBr fluorescence, so again you lose 10-20X sensitivity.
- To minimize waste, post-stain the gel in 0.5ug/ml EtBr solution (in water) for 15-30 min, let stand (wrapped in plastic for 15-30 minutes and then image). If background is high destain in water or 1mM $MgSO_4$ for 10 minutes.
- Other stains: SYBR Green, silver, SYBR Gold – see Molecular Probes handbook



Software programs use size standard length and mass to provide approximations for your unknown bands.

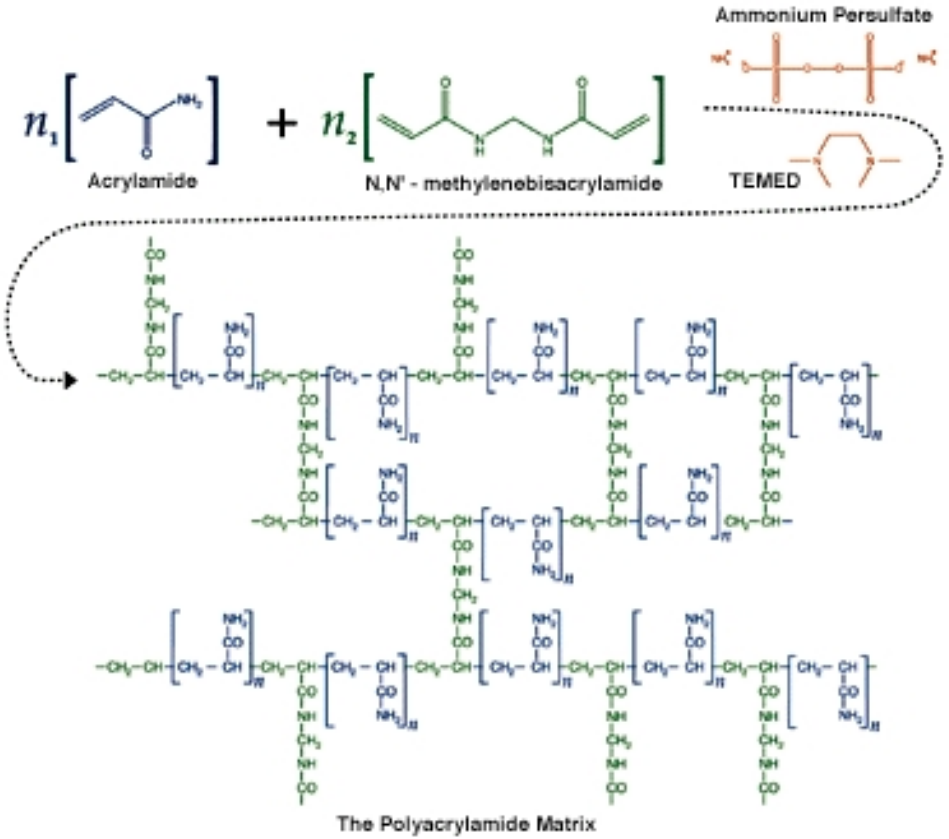
The migration here shows why you should include size standards on both edges (at least)



Preparation notes and variations for Agarose gels follow.

- Best band resolution comes with thin combs (<1mm) and wide teeth. (clean everything!) and very thin buffer layer.
    - Use 1X TBE for best resolution of DNA <12kbp, if you are not recovering of processing the DNA (use 1X TAE in that case)
    - Let agarose powder hydrate for ~2min before heating – add powder to buffer slowly, with swirling, to prevent clumping.
    - Heat in the microwave on high at 20 sec intervals, swirling between.
    - Cool to 50-60C before pouring – try to avoid bubbles. Add ethidium bromide just prior to pouring if using it during the run.
    - Let set 30 minutes before using.
- Denaturing methods:
    - Alkaline gels for ssDNA: Just prior to pouring the gel (and it must be 50C or less) add 50X alkaline buffer (50X = 1.5M NaOH/50mM EDTA) – use this for the running buffer as well, and use a loading dye of 60mM NaOH/2mM EDTA/20% Ficoll/0.06% Bromocresol Green).
    - Formaldehyde gels for RNA: use water treated with DEPC. Run in 10X MOPS buffer/3% formaldehyde (of a 37% solution) – add to cooled agarose, pour and run in a hood.

Acrylamide gels use a polymer with controlled levels of cross-linking to yield more precise separation of molecules.



Polyacrylamide Gel Applications

*Recommended applications for each formulation are shown in **bold***

Acrylamide:MBA Ratio	Gel %	Native DNA/RNA (bp)	Denatured DNA/RNA (bp)	Protein (kd)
19:1	4	100-1500	**70-500**	100-200
"	6	60-600	**40-400**	40-150
"	8	40-500	**20-200**	20-100
"	10	30-300	**15-150**	15-70
"	12	20-150	**10-100**	8-60
29:1	5	**200-2000**	**70-800**	>150
"	6	**80-800**	**50-500**	50-200
"	8	**60-400**	**30-300**	30-125
"	10	**50-300**	**20-200**	20-100
"	12	**40-200**	**15-125**	10-70
"	20	**<40**	**<40**	<30
37.5:1	6	.	.	**60-200**
"	8	.	.	**50-150**
"	10	.	.	**25-100**
"	12	.	.	**15-80**

Polyacrylamide Gels

Recommended Polyacrylamide % for Separation in Denaturing Gels

% Acrylamide	Effective Range of Separation (nucleotides)
3.5	>500
5	151-500
10	61-150
15	30-60
20	<30

Dye Migration in Denaturing Polyacrylamide Gels

% Acrylamide	Xylene Cyanol (nucleotides)	Bromophenol Blue (nucleotides)
5	130	35
6	106	26
8	75	19
10	55	12
12	28	8

Dye Migration in Nondenaturing Polyacrylamide Gels

% Acrylamide	Effective Range of Separation (bp)	Xylene Cyanol (nucleotides)	Bromophenol Blue (nucleotides)
3.5	100-1000	460	100
5	100-500	260	65
8	60-400	160	45
12	50-200	70	20
15	25-150	60	15
20	5-100	45	12