Chem 419 (2nd half) and 519
Bioanalytical chemistry

1. Direct detection and quantitation of biomolecules in solution

Suggested reading: **Section 1.1 to 1.3** of
Mikkelsen and Cortón, *Bioanalytical Chemistry*

Source Material
- Chapters 4 and 12 of Introduction to Genetic Analysis Anthony: J.F. Griffiths, Jeffrey H. Miller, David T. Suzuki, Richard C. Lewontin, William M. Gelbart (courtesy of the NCBI bookshelf).
- Chapters 4 and 6 of Biochemistry: Berg, Jeremy M.; Tymoczko, John L.; and Stryer, Lubert (courtesy of the NCBI bookshelf).
- Chapters 3 and 7 of Molecular Cell Biology: Lodish, Harvey; Berk, Arnold; Zipursky, S. Lawrence; Matsudaira, Paul; Baltimore, David; Darnell, James E. (courtesy of the NCBI bookshelf).
- The Molecular Probes Handbook from Invitrogen (http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook.html)
- Other sources mentioned on specific slides

Determining DNA concentration:
Ultraviolet absorbance

(A)
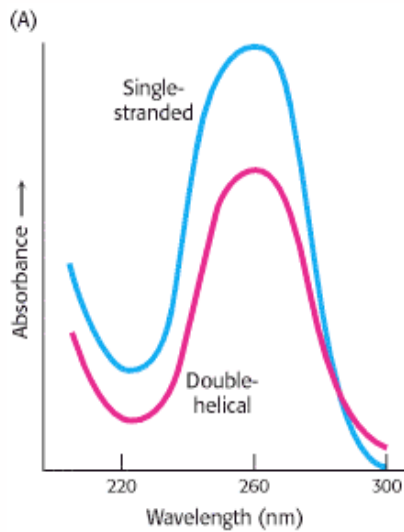
Absorbance of nucleotides at 260 nm

	MW	λ_{max}	ε $(M^{-1}cm^{-1})$
ATP	507.2	259	15,400
CTP	483.2	271	9,000
GTP	523.2	253	13,700
UTP	484.2	262	10,000

Average weight of a DNA basepair (sodium salt) = 650 daltons

1.0 A_{260} unit ds DNA = 50 μg/ml = 0.15 mM (in nucleotides) = 0.075 mM in basepairs
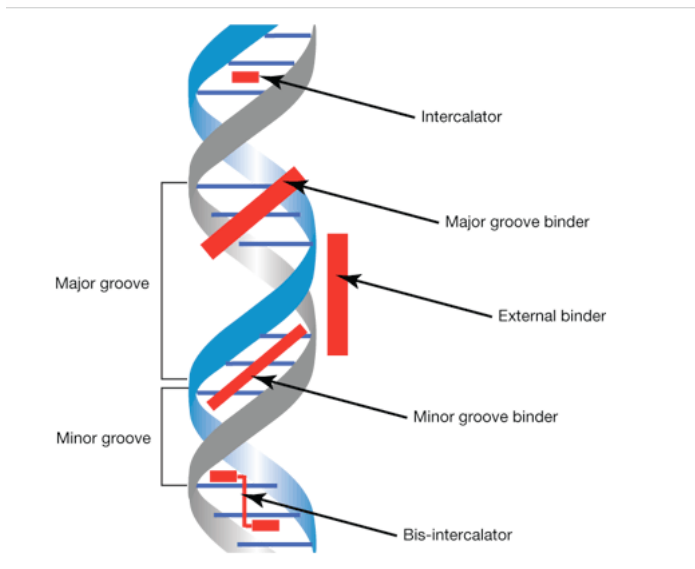1.0 A_{260} unit ss DNA = 33 μg/ml = 0.10 mM (in nucleotides)
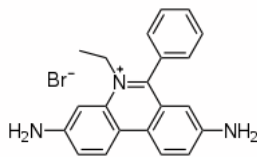1.0 A_{260} unit ss RNA = 40 μg/ml = 0.11 mM (in nucleotides)

http://www.neb.com/nebecomm/tech_reference/default.asp?

- The simplest way of determining the concentrations of either nucleic acids or proteins is to use UV absorbance measurements and then use the Beer-Lambert law with theoretical extinction coefficients. In addition, it is a non-destructive technique and the sample is easily recovered.
- DNA has a distinct absorbance band at 260 nm, while proteins have a distinct absorbance at 280 nm. In practice, these absorbance bands are broad and overlap with each other. Regardless, the ratio of A260/A280 can be used as a criterion of the purity of protein or nucleic acid samples during their purification. A ratio of 2 or greater indicates that the DNA is relatively free of proteins.
- Note that for many biomolecules, the idea of purity is arbitrary. It is often more accurate to say that a biomolecule has been 'enriched' rather than 'purified'. What really matters is that the biomolecule is 'pure enough' for downstream applications.
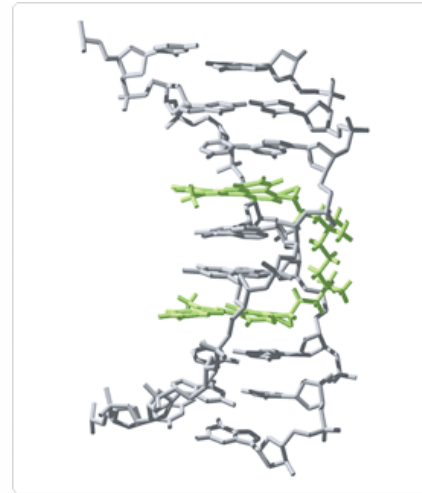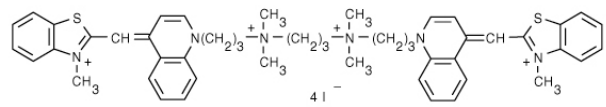
There are a wide variety of synthetic dyes that[3] increase fluorescence when bound to dsDNA

Intercalator

Major groove binder

External binder

Minor groove binder

Bis-intercalator

Major groove

Minor groove

ethidium bromide, an intercalator

Toto-1, a bis-intercalator

- The two most common types of 'classic' reagents for quantifying DNA in solution are Intercalating dyes (e.g., ethidium bromide and propidium iodide) and minor-groove binders (e.g., DAPI, SYBR green) and the Hoechst dyes)
- The Toto series of dyes are covalent dimers of the fluorescent dyes known as cyanines. They have very high affinity for DNA due to the ability to interchelate twice per molecule. They are also very bright fluorophores, enabling detection of lower concentrations of DNA. The Toto-type dyes are available in a wide-variety of colors.
- One drawback of ethidium bromide is that it is carcinogenic and must be handled quite carefully
- There are a number of additional synthetic dyes which are supposedly less carcinogenic. Molecular probes cells one called 'SYBR Safe' which has apparently been thoroughly tested for carcinogenicity and found to be relatively safe. This kit probably contains the SYBR green dye.
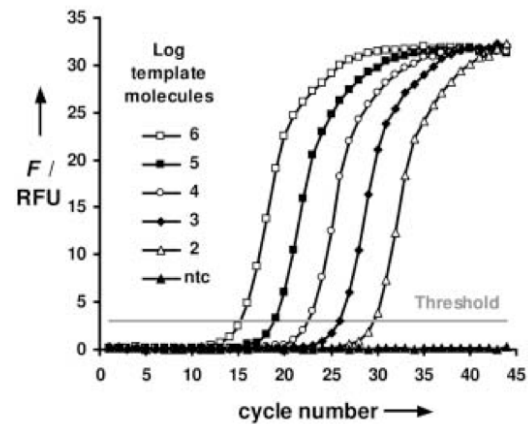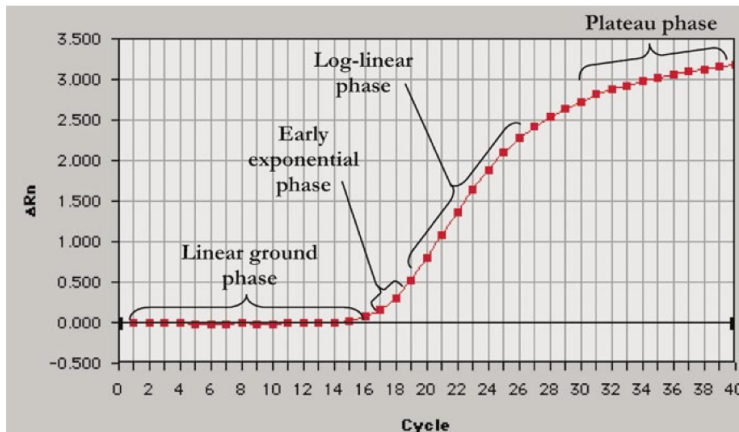
DAPI: a minor-groove binder
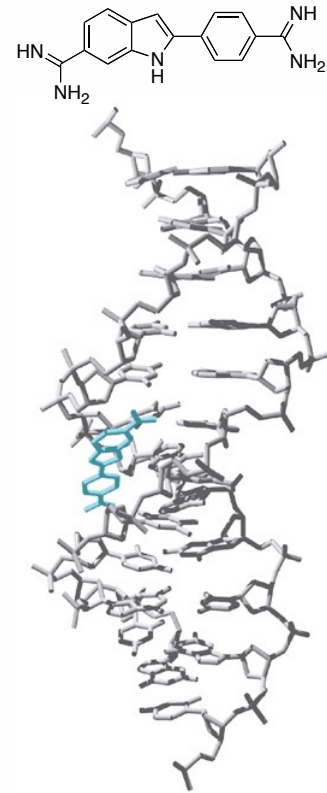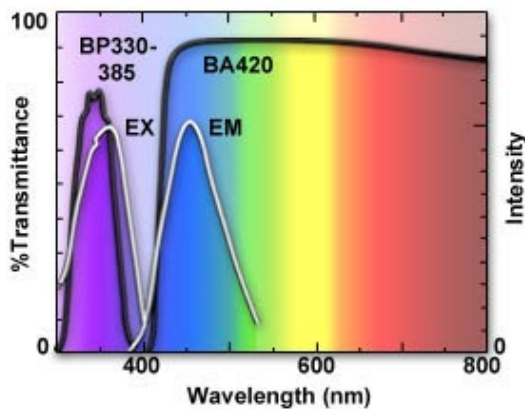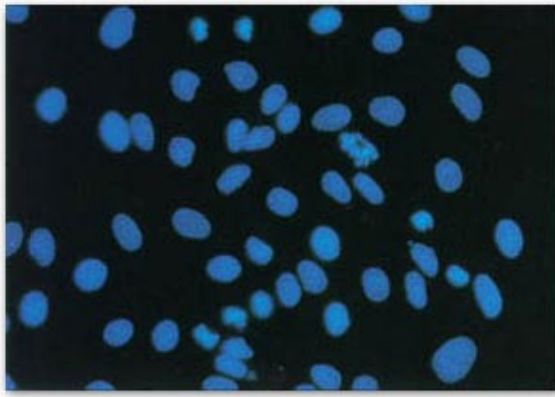
micro.magnet.fsu.edu

- The blue fluorescent 4'-6-diamidino-2-phenylindole (DAPI) nucleic acid stain preferentially stains dsDNA it appears to associate with AT clusters in the minor groove. Binding of DAPI to dsDNA produces a ~20-fold fluorescence enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove.
- DAPI also binds RNA, however in a different binding mode — one thought to involve AU-selective intercalation. The DAPI/RNA complex exhibits a longer-wavelength fluorescence emission maximum than the DAPI/dsDNA complex (~500 nm versus ~460 nm) and a quantum yield that is only about 20% as high.
- DAPI is widely used for staining of DNA in cells. For interphase cells, essentially the whole nucleus becomes blue fluorescent due to the binding of DAPI in the minor groove of the genomic DNA.
- In addition to the absorbance and emission of DAPI, the spectrum in the bottom left shows the transmission profile of an excitation and emission filter that would be used in the imaging of DAPI.
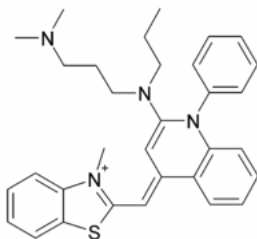
Determining DNA concentration:
Real-time PCR

- Under typical PCR conditions with many (i.e., >30) cycles the reaction rate plateaus as primers and dNTPs are depleted and the number of templates approaches the number of Taq enzyme molecules. This means that the final amount of DNA produced ends up being relatively independent of the amount of template initially added.
- However, at fewer number of cycles, the total concentration of DNA in the PCR tube is highly sensitive to the original number of template molecules. The reason is that the growth is exponential and so the a 2-fold difference in number of template molecules will be preserved throughout the earliest cycles of amplification. At the logical extreme, the difference between 1 and 2 molecules should be just as easily detectable as the difference between 500 and 1000. But how do we know how much product has been produced in the PCR tube at the early cycles?
- This is done with 'real-time' PCR where the real-time part refers to the fact that the tube is, in effect, a cuvette, and the PCR machine is also a fluorescence spectrophotometer. The fluorescence intensity of the sample in the test tube is monitored in real time. RT-PCR is used almost exclusively to detect cDNA molecules produced from reverse transcription of mRNA with the ultimate goal of determining the original concentration of mRNA. This leads to confusion (at least in my mind) because both real time and reverse transcription are abbreviated RT and they are often used together!
- THEORY OF REAL-TIME PCR [Quoted from Marisa L. Wong and Juan F. Medrano 'Real-time PCR for mRNA quantitation' BioTechniques 39:75-85 (July 2005). PCR can be broken into four major phases (Figure shown on LHS of slide): the linear ground phase, early exponential phase, log-linear (also known as exponential) phase, and plateau phase. During the linear ground phase (usually the first 10–15 cycles), PCR is just beginning, and fluorescence emission at each cycle has not yet risen above background. Baseline fluorescence is calculated at this time. At the early exponential phase, the amount of fluorescence has reached a threshold where it is significantly higher (usually 10 times the standard deviation of the baseline) than background levels. The cycle at which this occurs is known as Ct in ABI Prism® literature (Applied Biosystems, Foster City, CA, USA) or crossing point (CP) in LightCycler® literature (Roche Applied Science, Indianapolis, IN, USA) (2,10). This value is representative of the starting copy number in the original template and is used to calculate experimental results. During the log-linear phase, PCR reaches its optimal amplification period with the PCR product doubling after every cycle in ideal reaction. Finally, the plateau stage is reached when reaction components become limited and the fluorescence intensity is no longer useful for data calculation.
- *Q: Can you explain a little more about how real time PCR is used in Detection rather than quantification?*
- A: There isn't really any difference between the two applications. Of course, if the target DNA sequence is not present, you would not expect to get any product in the PCR amplification (whether it is normal PCR or real time PCR).

Probes for detecting DNA during real-time PCR

A. DNA binding dyes

B. Hybridization probes
4 oligo method 3 oligo method
2 primers 2 probes 2 probes 1 primer
A A
D D
D D

C. Hydrolysis probes
R Q
R Q

D. Molecular beacons
R Q
R Q

E. Scorpions
R Q
Q

F. Sunrise primers
Q R
R
Q

G. LUX primers

Q Quencher fluorophore
R Reporter fluorophore
A Acceptor fluorophore
D Donor fluorophore
● Quenched fluorophore
☾ DNA polymerase
🚫 DNA polymerase blocker
✹ Light-emitting fluorescent fluorophore

SYBR green: a dye that increases in fluorescent intensity 1000x when bound to the minor groove of DNA

Fluorescence excitation — Fluorescence emission
Wavelength (nm)
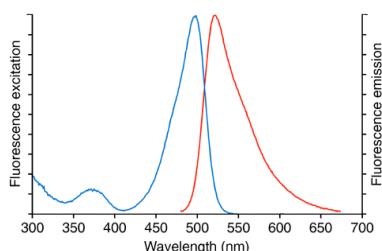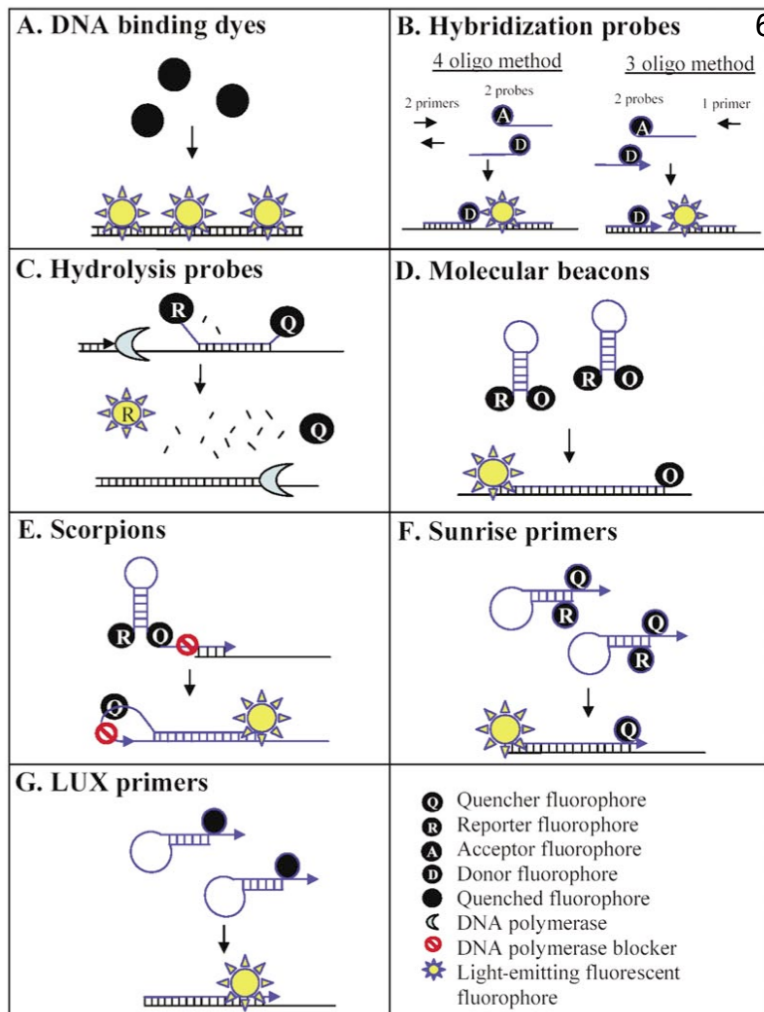300 350 400 450 500 550 600 650 700

Image sources:
*BioTechniques* 2005, **39**:75-85.
Invitrogen, Wikipedia

- The next question becomes, how do we detect the total amount of DNA produced using fluorescence?
- There are a variety of creative solutions to this problem, many of which are schematically represented on this slide. Many of these strategies use the phenomenon of fluorescence resonance energy transfer in order to quench the fluorescence of the donor or increase the fluorescence of the acceptor.
A. The simplest detection strategy to use is to include a dye that increases its fluorescence when bound to dsDNA
B. Hybridization probes work by positioning a donor and acceptor fluorophore close together using labeled probes.
C. Hydrolysis probes rely on the 5',3'-exonuclease activity of the DNA polymerase to destroy the probe and separate the reporter fluorophore from a quencher.
D. Molecular beacons hybridize to newly synthesized DNA and, in doing so, disrupt a stem-loop structure that had originally positioned the reporter fluorophore close to a quencher.
E. Scorpions are a bit tricky to explain. It is essentially a molecular beacon attached to a primer as shown in the figure. The red circle with a line through it represents a nucleotide that can not serve as a template. So DNA synthesis is blocked when it reaches this point (in later cycles where the Scorpion-containing DNA strand is used as a template. The hairpin portion is designed to anneal to the adjacent region of newly synthesized DNA. It is this annealing that separates the quencher from the fluorophore.
F. Sunrise primers are sort of like Scorpions but with a somewhat simpler mechanism. In the double stranded PCR product that quencher is far away from the donor.
G. LUX primers include a dye that only fluoresces when the nucleotide it is attached to is incorporated into double stranded DNA. It has been found that certain dyes at the end of a hairpin structure can be quenched by certain sequences of nucleotides. In a sense, the hairpin itself is the quencher in this detection strategy.
● Q: I did not understand the difference between 2 cases in Hybridization probes in DNA determining. In one case, Is Donor on the primer which continues DNA duplication and Acceptor on DNA which just hybridize to DNA? And the other case both of them are primer? Is it possible (because DNA synthesis just goes from 5' to 3')?
● A: In the 4 oligo method, the acceptor and donor are on probe molecules that are not primers. These probe molecules very likely lack a 3' hydroxyl at their 3' ends so that the DNA can not be further extended. In the case of the 3-oligo method, the primer with the donor does have a 3' hydroxyl, so it can be used as one of the primers for amplification. Note that it would not be possible to have a "2 oligo method" where both primers were also the probe molecules. The reason is that the primers need to bind to opposite ends of the template and so the donor and acceptor fluorophores would not be close to each other.
●Q. For P. 6, I don't quite understand the function of DNA polymerase blocker in the Scorpions structure. From my understanding, if the blocker is absent from the structure, and when the Scorpion-containing DNA serves and a template, the DNA polymerase will synthesize a new strand of complementary DNA. By then, the Scorpion-containing DNA template will be stretched out and the reporter and quencher will be far away from each other, so the reporter should be able to emit light as well.
●A. You are correct about what would happen if the blocker (an abasic site) was not present. Essentially the Scorpion primer would be a Sunrise primer in this case, and there would still be an unquenching of the fluorescence. The Scorpion primer is just another way of getting this separation and it does not necessarily have any major advantages or disadvantages relative to the Sunrise primer. The product page at Sigma seems to emphasize the fact that the key interaction is intramolecular rather than intermolecular (as in the case of Sunrise primers), and this results in a faster rate of quenching when cooling from the melting to the annealing temperature.
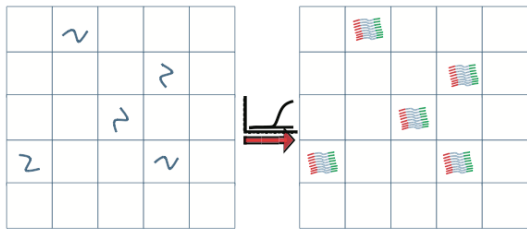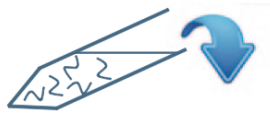
Digital PCR

In digital PCR, the DNA is highly diluted and dispensed into either 1000s of small wells or droplets.
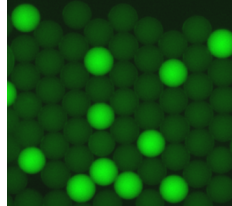
Primers, nucleotides, and polymerase are also present in the wells or droplets

Thermocycling causes amplification only in those wells or drops that have a copy of the template

The number of wells or drops in which amplifcation occurs (as detected using fluorescence) = the number of molecules in the original sample.
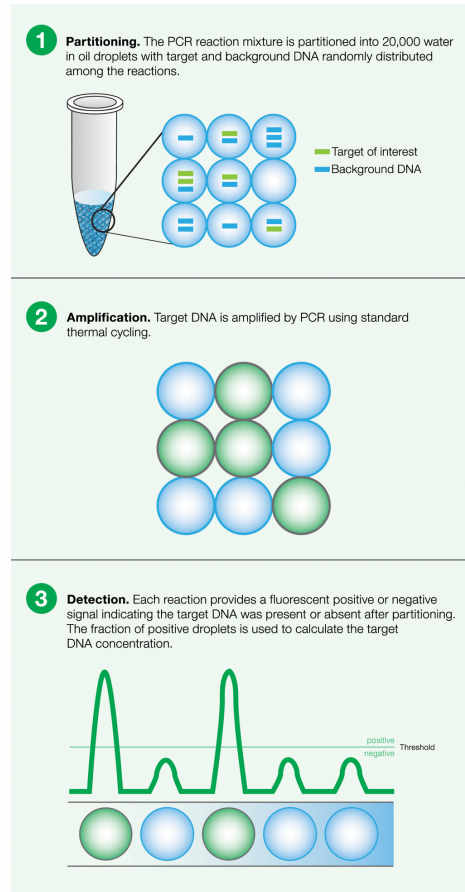
Can count DNA molecules in 10-100s range

TATAA Biocenter

1 **Partitioning.** The PCR reaction mixture is partitioned into 20,000 water in oil droplets with target and background DNA randomly distributed among the reactions.

Target of interest
Background DNA

2 **Amplification.** Target DNA is amplified by PCR using standard thermal cycling.

3 **Detection.** Each reaction provides a fluorescent positive or negative signal indicating the target DNA was present or absent after partitioning. The fraction of positive droplets is used to calculate the target DNA concentration.

positive
negative
Threshold

Figure 1. Principles of droplet digital PCR.

- http://www.nature.com/nmeth/journal/v9/n6/full/nmeth.2027.html
- http://media.invitrogen.com.edgesuite.net/ab/applications-technologies/real-time-pcr/digitalpcr/index.html
- http://weitzlab.seas.harvard.edu/research/zhang-huidan.html

Determining protein concentration: Ultraviolet absorbance
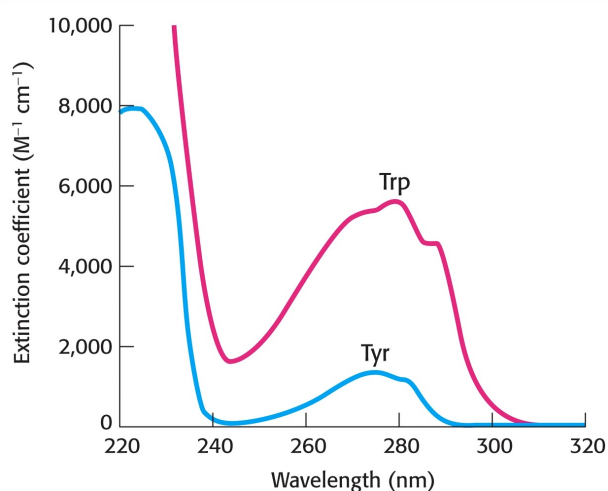
Amino acids with absorbance at 280 nm

Trp $5500\ M^{-1}cm^{-1}$ at 280nm
Tyr $1490\ M^{-1}cm^{-1}$ at 280nm
Cys-Cys $125\ M^{-1}cm^{-1}$ at 280nm - **note that this is per cystine (a.k.a disulfide)**

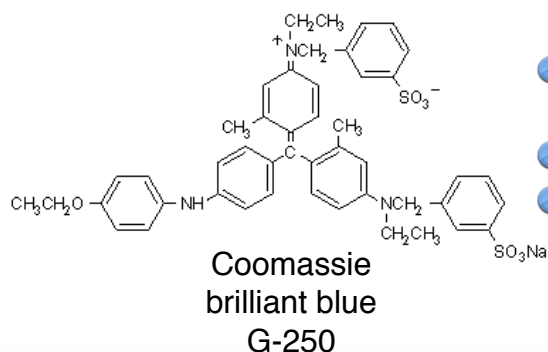In 6.0 M guanidium hydrochloride, 0.02 M phosphate buffer, pH 6.5.

Very good overview of protein concentration determination:
http://www-class.unl.edu/biochem/protein_assay/

- Determination of protein concentration by ultraviolet absorption (at 280 nm) depends almost entirely on the presence of aromatic amino acids in proteins. Tyrosine and tryptophan absorb at approximately 280 nm. Disulfides also absorb at 280 nm, but much more weakly. The real advantages of this method of determining protein concentration are that the sample is not destroyed and that it is very rapid.
- Different proteins will have different amino acid compositions and thus different molar absorptivity's. However, if you know the sequence of the protein, you can easily calculate a theoretical extinction coefficient based on the number of Trp, Tyr, and disulfide bonds in the protein. There are a number of online tools that can do this calculation for you.
- An even more quick and dirty method for estimating the protein concentration is to use [Protein] (mg/mL) = 1.55*A280 - 0.76*A260 . This method assumes that all proteins have more or less the same number of tryptophans and tyrosines.
- To make an even more accurate determination of protein concentration, you could produce a standard curve (A280) with known amounts of purified protein. You will also have to provide a blank that is appropriate for the sample and contains the same concentrations of buffer and salts as the sample. It is often convenient to dialyze the sample and measure the absorbance of the retentate (still in the dialysis sack) using the dialysate as the blank. Care must be taken to use quartz cuvettes, since glass absorbs UV light.
- For increased sensitivity, the wavelength can be lowered to the range of 210 to 225 nm. This measures the amide bond in proteins. However it is much more subject to interference from many more biological components and compounds used to make buffer solutions.
- In principle, you could also use the intrinsic fluorescence of your protein as a measure of protein concentration. Trp is the primary contributor to intrinsic fluorescence of proteins. Trp fluorescence is much less predictable than extinction coefficient, so the only way to do this with any degree of confidence would be to first make a standard curve with known concentrations of your protein.
- http://www.basic.northwestern.edu/biotools/proteincalc.html
- http://ca.expasy.org/tools/protparam-doc.html

Determining protein concentration:
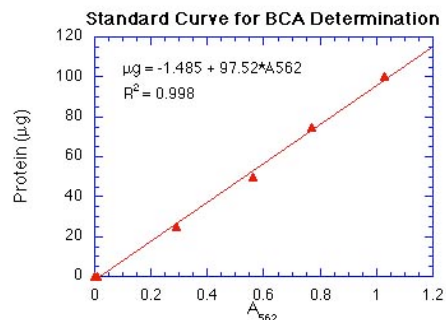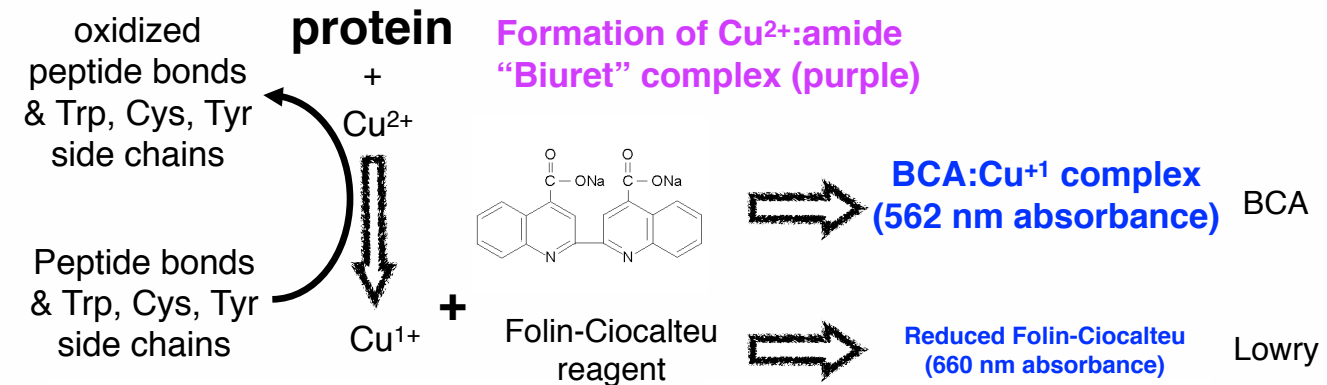# Bradford assay

- binds to positively charged (mostly Arg) and aromatic amino acids
- changes colour from pale red to blue
- protein complex has absorbance maximum at 590 nm

Coomassie brilliant blue G-250

- popular because it is fast (~5 min) and simple
- however, some disadvantages:
  - absorbance increase is non-linear
  - generally underestimates protein concentrations relative to other methods

Standard Curve with Bradford Assay

$$\mu g = -0.717 - 7.55*A + 19.6*A^2$$
$$R^2 = 0.992$$
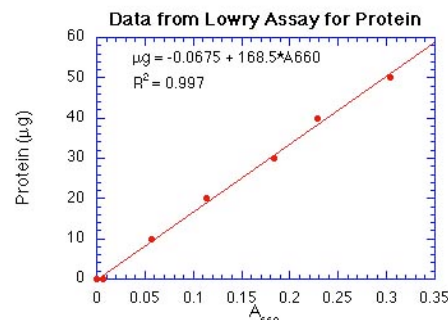
Protein (μg) vs A_{595}
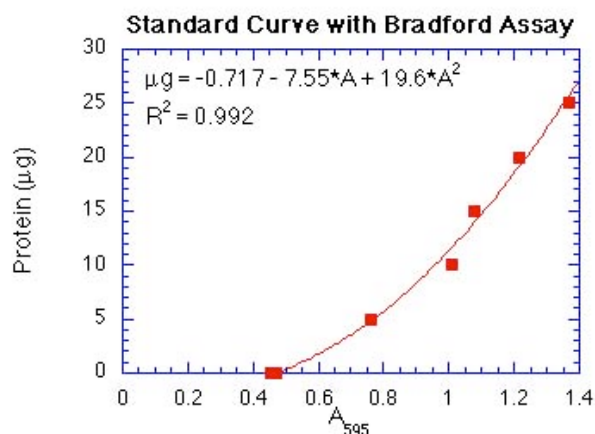
- The Bradford assay is a very popular protein assay method because it is simple, rapid, inexpensive and sensitive. The Bradford assay works by the action of Coomassie brilliant blue G-250 dye (CBBG). This dye specifically binds to proteins at arginine, tryptophan, tyrosine, histidine and phenylalanine residues. It should be noted that the assay primarily responds to arginine residues (eight times as much as the other listed residues) so if you have an arginine rich protein, you may need to find a standard that is arginine rich as well.
- CBBG binds to these residues in the anionic form, which has an absorbance maximum at 595 nm. The free dye in solution is in the cationic form, which has an absorbance maximum at 470 nm. The assay is monitored at 595 nm in a spectrophotometer, and thus measures the CBBG complex with the protein. It should be emphasized that the absorption spectra of the two forms of the dye overlap. This causes the assay to respond non-linearly in the standard curve.
- However, a standard curve is a close approximation of a straight line over relatively narrow ranges of concentrations.
- *Q. In the Bradford assay: would we be maintaining the protein mix in a buffer with a pH<pI in order to ensure the + charged macromolecule for CBBG anion binding? This was not discussed and unless we are relying on the 5 amino acids that CBBG targets to be + charged at neutral pH I don't see how this assay would be very sensitive.*
- *A. It's not necessary to think about the overall pI in this case, just the pKa of the individual amino acids. For example, arginine will always be protonated at any reasonable pH value.*

Determining protein concentration:
Lowry and BCA assay

oxidized peptide bonds & Trp, Cys, Tyr side chains

protein
+
Cu^{2+}

Formation of Cu^{2+}:amide "Biuret" complex (purple)

Peptide bonds & Trp, Cys, Tyr side chains

Cu^{1+}

+

BCA:Cu^{+1} complex (562 nm absorbance) BCA

Folin-Ciocalteu reagent

Reduced Folin-Ciocalteu (660 nm absorbance) Lowry

Standard Curve for BCA Determination

$\mu g = -1.485 + 97.52 \cdot A562$
$R^2 = 0.998$

Protein (μg)

A$_{562}$

Data from Lowry Assay for Protein

$\mu g = -0.0675 + 168.5 \cdot A660$
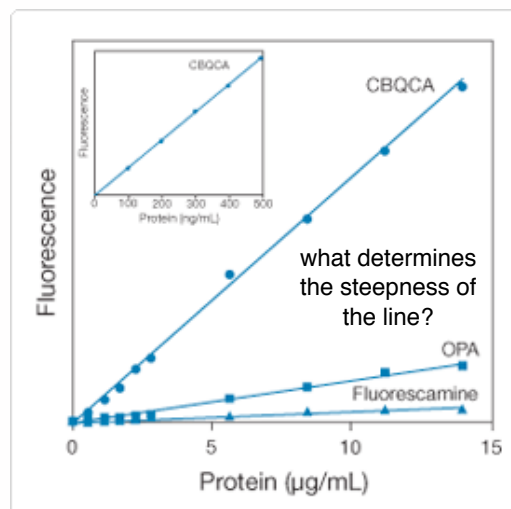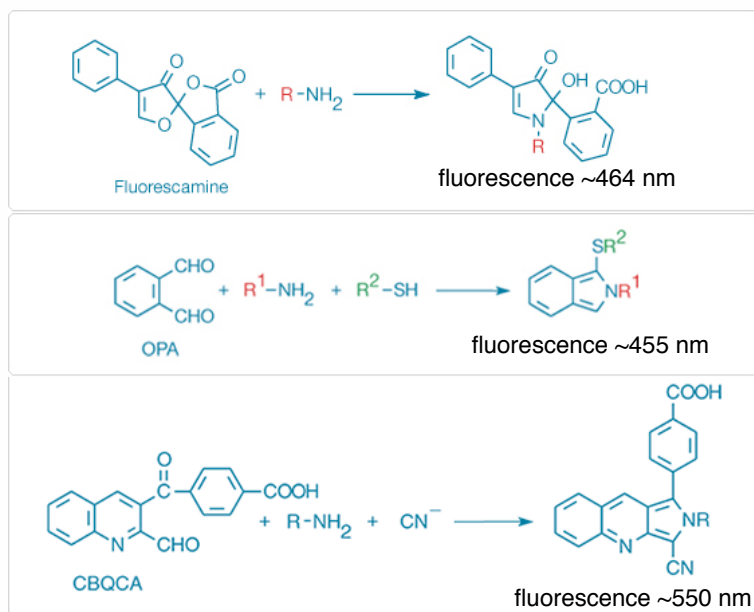$R^2 = 0.997$

Protein (μg)

A$_{660}$

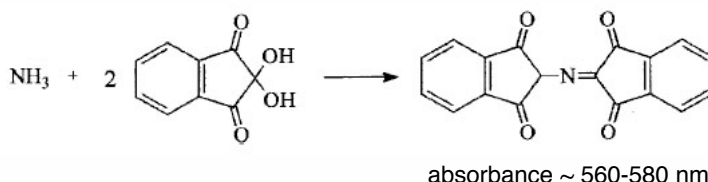🔴 BCA is generally the preferred method, though lipids can interfere

🔴 Lowry is not quite as popular as BCA, but better for protein samples with lipid contamination

- The Lowry method relies on two different reactions. The first is the formation of a copper ion complex with amide bonds. This is called a "Biuret" chromophore. The second is the reduction of Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate).
- The reduced Folin-Ciocalteu reagent is blue and thus detectable with a spectrophotometer in the range of 500-750 nm. The Biuret reaction itself is not all that sensitive. Using the Folin-Ciocalteu reagent to detect reduced copper makes the assay nearly 100 times more sensitive than the Biuret reaction alone. The assay is relatively sensitive, but takes more time than other assays and is susceptible to many interfering compounds.
- Many substances are known to interfere with the Lowry assay: detergents, carbohydrates, glycerol, Tricine, EDTA, Tris, potassium compounds, sulfhydryl compounds, disulfide compounds, magnesium and calcium. Most of these interfering substances are commonly used in buffers for preparing proteins. This is one of the major limitations of the assay.
- The Lowry assay is sensitive to variations in the content of tyrosine and tryptophan residues. If the protein you are assaying has an unusual content of these residues, an appropriate substitute standard is required. The standard curve is linear in the 1 to 100 µg protein region. Most researchers use 660 nm, but other wavelengths also work and may reduce the effects of contamination (e.g. chlorophyll in plant samples interferes at 660 nm, but not at 750 nm).
- The BCA assay is similar to Lowry assay, except that the Folin-Ciocalteu reagent used in the Lowry assay is replaced with bicinchoninic acid (BCA). The BCA reagent forms a complex with Cu+1, which has a strong absorbance at 562 nm. BCA is advantageous in that it does not interact with as many substances as the Folin-Ciocalteu reagent, especially detergents and buffers. The BCA assay is limited in that it interacts with most reducing agents and copper chelators. In general, these are not critical components of buffers and can be easily eliminated prior to assay.

Amine-reactive methods

Fluorescamine

fluorescence ~464 nm

OPA

fluorescence ~455 nm

CBQCA

fluorescence ~550 nm

what determines the steepness of the line?

CBQCA

OPA

Fluorescamine

Fluorescence vs Protein (µg/mL)

Ninhydrin method

Protein is first completely hydrolyzed (6 M HCl, 100 C, 24 h) to liberate ammonia from the peptide bonds.

NH_3 + 2 [indanetrione·hydrate] → [Ruhemann's purple structure]

absorbance ~ 560-580 nm

http://probes.invitrogen.com/handbook/sections/0902.html

- Other fluorescent assays involve dyes that specifically react with primary amines (lysines and N-terminus). The main source of variation would be number of exposed lysine residues. One example is CBQCA. Note that this is a variation on classic and widely available method for detecting amines. Molecular Probes has improved upon the classic reagents and made it work somewhat better (i.e. longer wavelength, lower background)
- In general, fluorescence is 100's to 1000's of times more sensitive than absorbance for any quantification, including proteins.

The NanoOrange assay is a proprietary protein assay kit sold by Molecular Probes (Invitrogen)

Table 1. Comparison of methods for quantitating proteins in solution.

Assay Method	Useful Range	Comments
NanoOrange assay	10 ng/mL to 10 µg/mL	• Samples can be read up to six hours later without any loss in sensitivity • Low protein-to-protein signal variability • Detection not influenced by reducing agents or nucleic acids
BCA method [1]	0.5 µg/mL to 1.5 mg/mL	• Samples must be read within 10 minutes • Not compatible with reducing agents
Bradford assay [2]	1 µg/mL to 1.5 mg/mL	• Proteins precipitate over time • High protein-to-protein signal variability • Not compatible with detergents
Lowry assay [3]	1 µg/mL to 1.5 mg/mL	• Lengthy, multistep procedure • Not compatible with detergents, carbohydrates or reducing agents
Absorbance at 280 nm [4]	50 µg/mL to 2 mg/mL	• High protein-to-protein signal variability • Detection influenced by nucleic acids and other contaminants

not exactly insightful instructions...

Add dye to samples → Heat 10 minutes → Cool to room temperature → Read fluorescence
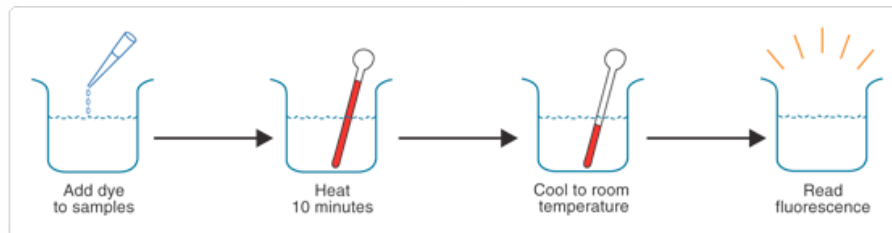
- Molecular probes (and others) have introduced additional fluorescence-based assays for determining protein concentration.
- Nanoorange is a patented technology and Molecular probes does not reveal the structure in their literature (http://probes.invitrogen.com/handbook/sections/0902.html)
- One of the big advantages of NanoOrange (as claimed by the manufacturer) is little protein-to-protein variation. How could this be possible?