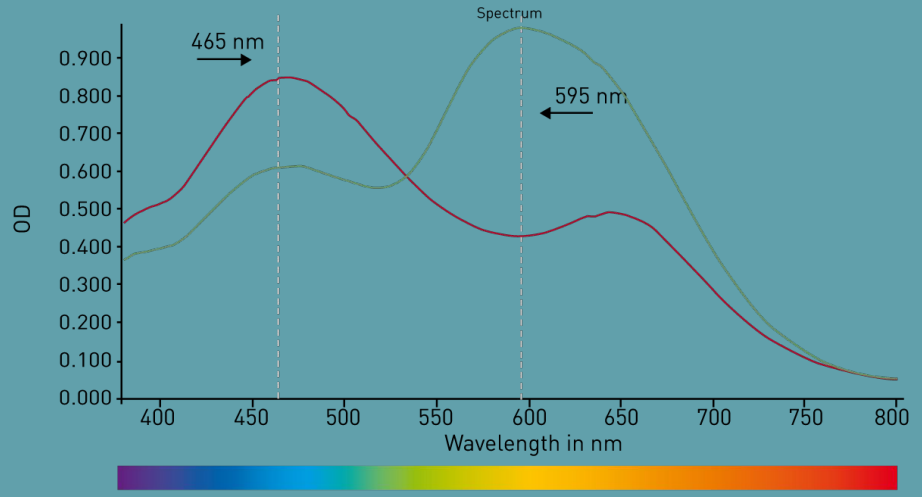
Measurement & The Standard Curve



Lost & Found - HELD 315
Please write your name on the bottom of the water bottles/cups and side of the safety goggles !!!

Safety

- Food/water bottles zipped up in backpacks (or outside)
- Keep stools always pushed in when standing
- Backpacks hanging up
- Goggles on- if they fog up you can step out to wipe them off
- Gloves worn while working with solutions
- Do not move or unplug the spectrophotometers
- All pipet tips should be ejected into the tip waste bin on your bench.
- Bradford Reagent is a corrosive acid- All Bradford reagent should be poured into the acid waste container on the side bench. Remember to put the lid back on when you are done.
- Rinse your plastic cuvettes in the sink and place them in the trash can in the front of the room.

Lab Objectives

- Dilute a concentrated stock solution to a working concentration.
- Accurately and precisely use pipettes in a laboratory setting.
- Use a spectrophotometer.
- Generate and evaluate a standard curve.
- Use a standard curve to determine the concentration of an unknown solution.

What we will be doing today?

- TODAY'S WORK: Measuring unknown protein concentration of 2% milk
- To do this we must address:
  - How to measure protein concentration?
    - Bradford protein assay
    - color changes (reddish to blue) with protein concentration
  - How to measure color change?
    - Spectrophotometer (set the absorbance @595nm)
  - How to conduct Bradford Assay?
    - Create a standard set of samples based of **known** protein concentration using BSA (Bovine Serum Albumin)
    - Plot the standard and analyze linear correlation ($y=mx+b$; R^2 value)
    - Compare **unknown** samples to standard set

Fundamental Knowledge

What are Dilutions?

- lowering the concentration of a solute in a solution by adding more solvent to the solution

How do we make dilutions?

- Example - a 1:10 dilution, 9 units of water and 1 unit of milk.

Mathematically this relationship can be shown by equation; c1v1 = c2V2

- Compare the mass in solution before and after dilution

What is a standard curve?

- graph of light absorbance versus solution concentration which can be used to figure out the solute concentration in unknown samples.

A serial dilution

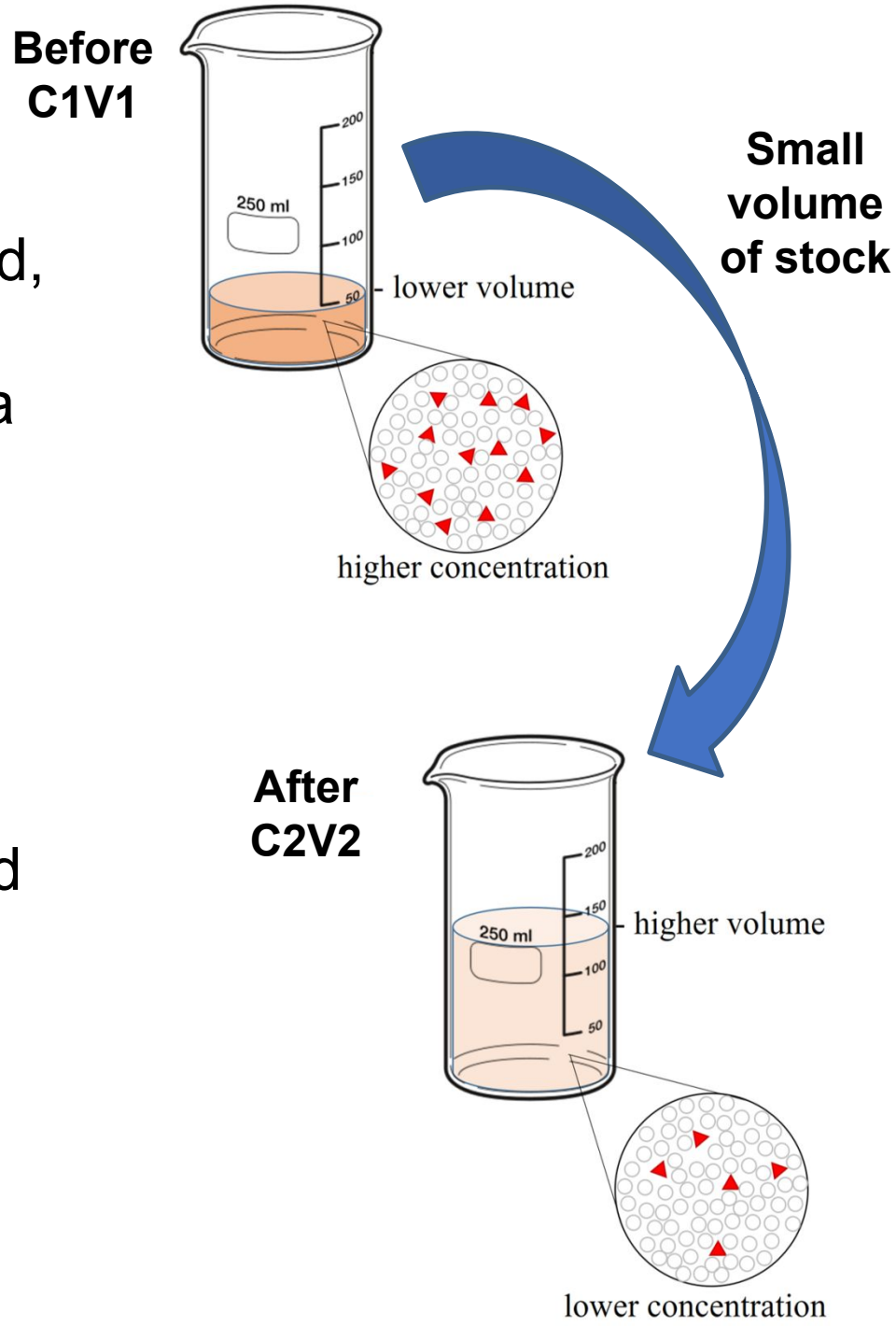
.- is a step-wise series of dilutions, where the dilution factor stays the same for each step. But concentration decreases by the same quantity in each successive step.

Dilutions

- Starting with a stock solution, highly concentrated, you will need to be able to calculate how much solvent and solute to mix to create a solution at a lower concentration.

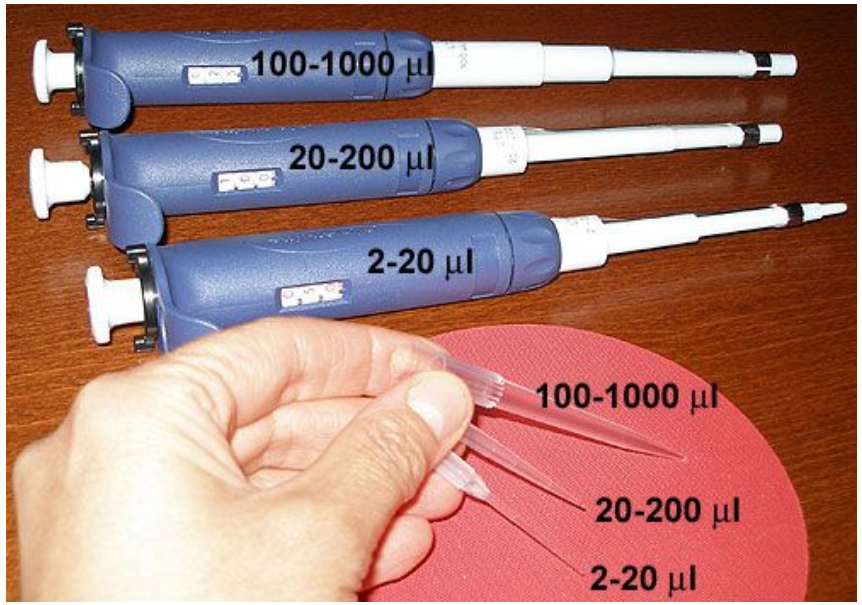
Equation C1V1 = C2V2

- What you have:
  - C1= concentration of stock solution
  - V1 = volume of stock solution you need to add to create a new solution at a lower concentration
- What you want:
  - C2= usually the concentration you want
  - V2= usually the final volume you want

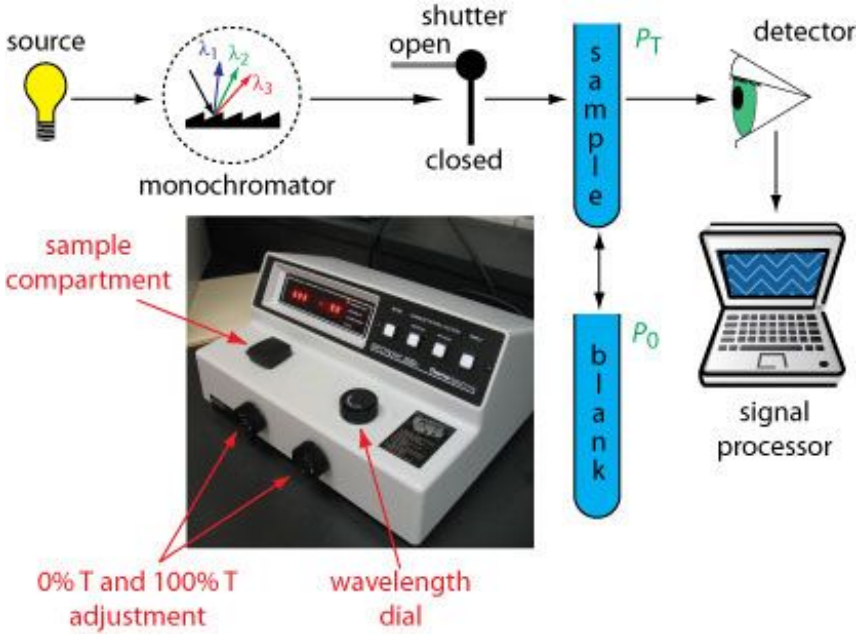


Lab Equipment

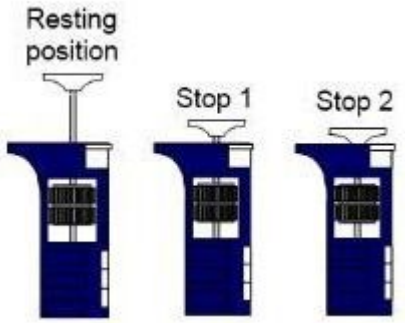
- **Micropipette:** an instrument used to measure/deliver amounts of liquid with high <u>accuracy</u> and <u>precision</u>



- **Spectrophotometer**: is an instrument that measures the number of photons (the intensity of light) absorbed after it passes through sample solution. With the spectrophotometer, the amount of a known chemical substance (concentrations) can also be determined by measuring the intensity of light detected.



How to Pipette

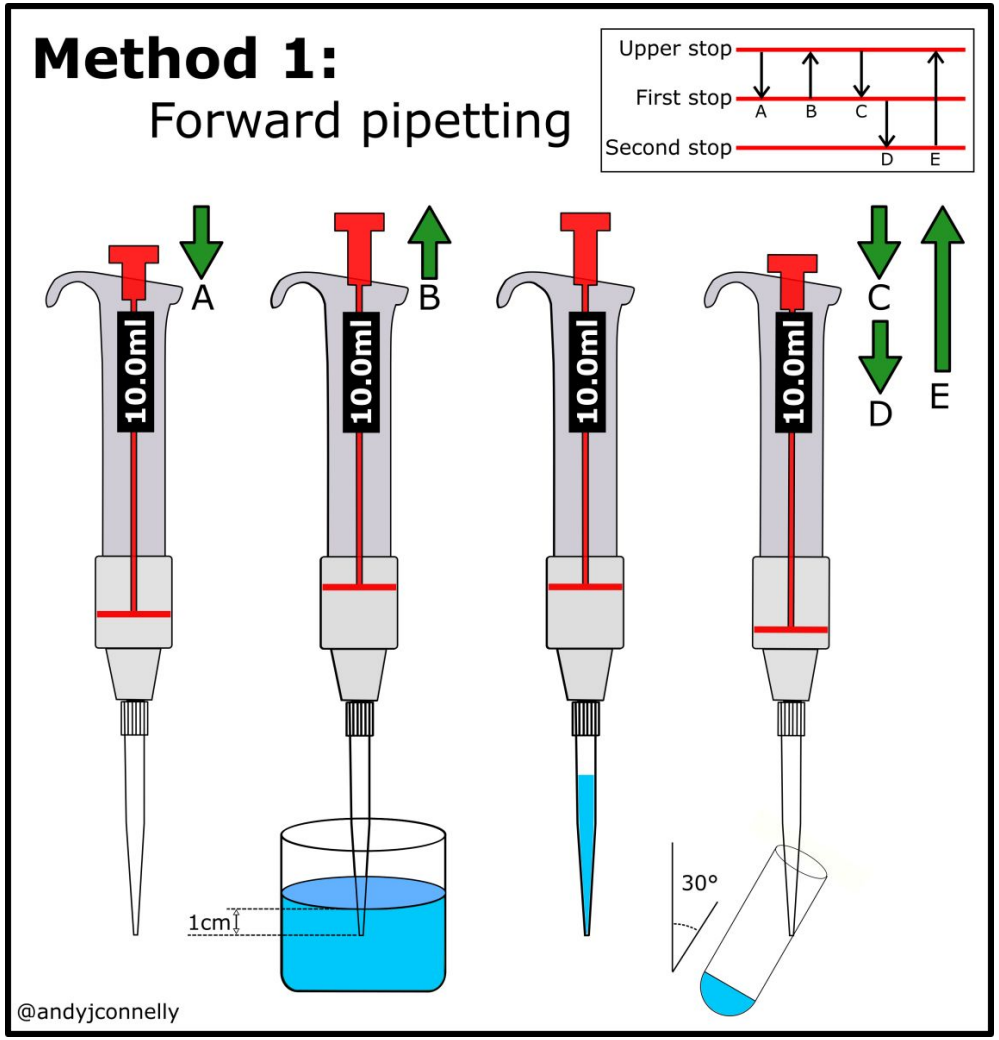


To drawn up the liquid,

1. Hold the pipette vertically; depress the plunger button to the first stop (A).
2. Place the tip just under the surface of the liquid (see the diagram)
3. Smoothly release the plunger button (B) keeping the tip at a constant depth.
4. Carefully withdraw the tip from the liquid, touching against the edge of the container to remove excess.

To dispense the liquid,

1. hold the tip at an angle of around 30-45º against the wall of the receiving container. Depress the plunger button to the first stop (C) and hold for one second.
2. Push the pipette to the second stop (D) while sliding the pipette tip against the walls of the container.



A spectrophotometer measures the number of photons (the intensity of light) absorbed after it passes through sample solution.

With the spectrophotometer, the amount of a known concentrations can also be determined by measuring the intensity of light detected.

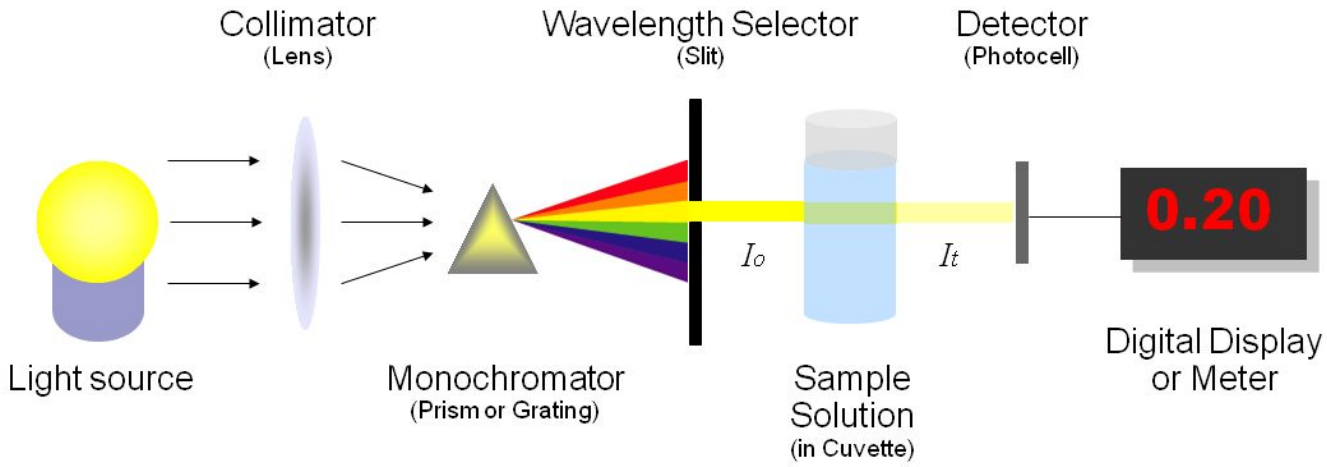
Depending on the range of wavelength of light source, it can be classified into two different types:

- **UV-visible spectrophotometer** uses light over the ultraviolet range (185 - 400 nm) and visible range (400 - 700 nm) of electromagnetic radiation spectrum (what we are using today).
- **IR spectrophotometer**: uses light over the infrared range (700 - 15000 nm) of electromagnetic radiation spectrum.

with the amount of absorbance known, you can determine the unknown concentration of the sample by using Beer-Lambert Law.

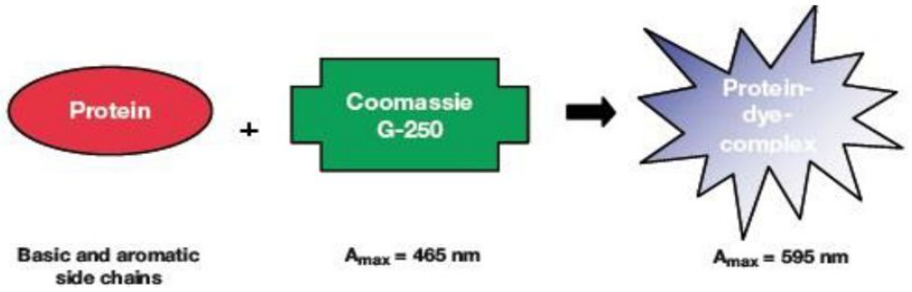
$A=\epsilon lc$

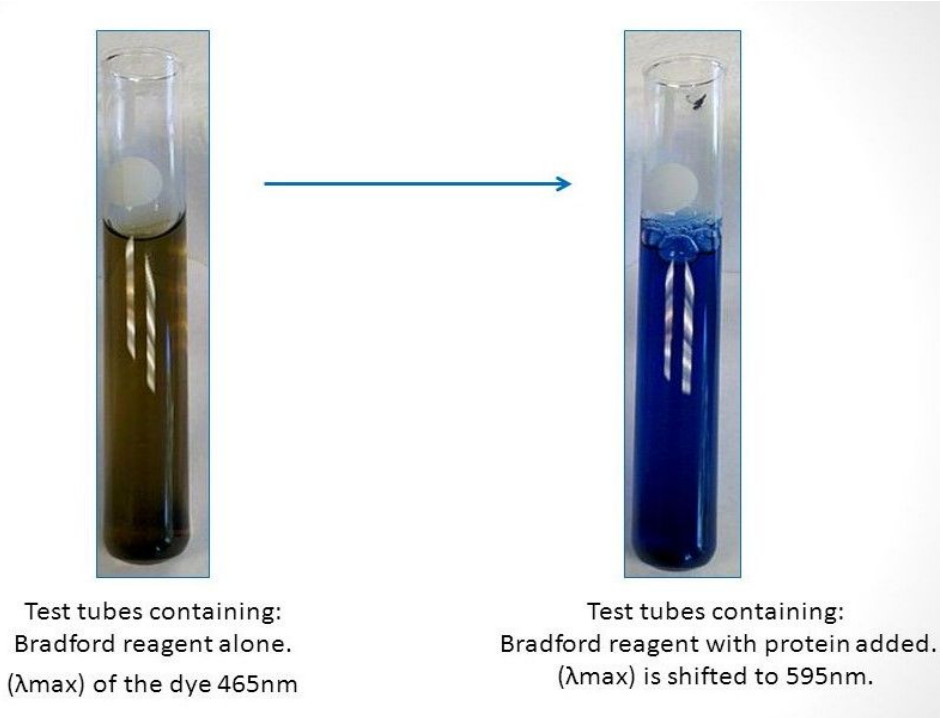
- A is the measure of absorbance (no units)
- ϵ is the molar extinction coefficient or molar absorptivity (or absorption coefficient)
- l is the path length
- c is the concentration



Bradford Assay

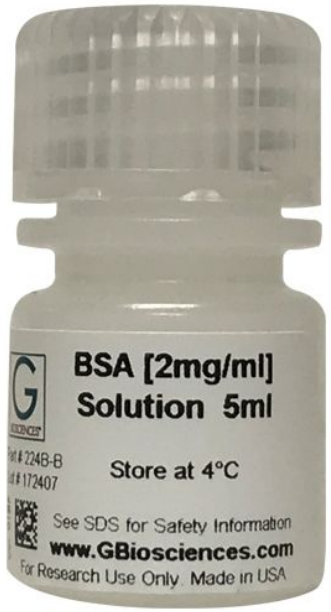
- The Bradford reagent consists of the dye Brilliant Blue G.
- This method relies on forming a complex by the binding of the dye Coomassie Brilliant Blue G-250 to the proteins. The absorption at 595 nm is proportional to the amount of protein present in the sample.
- The dye reagent reacts primarily with arginine residues and less so with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues.
- The practical advantages of the method are that the reagent is simple to prepare and that the color develops rapidly and is stable

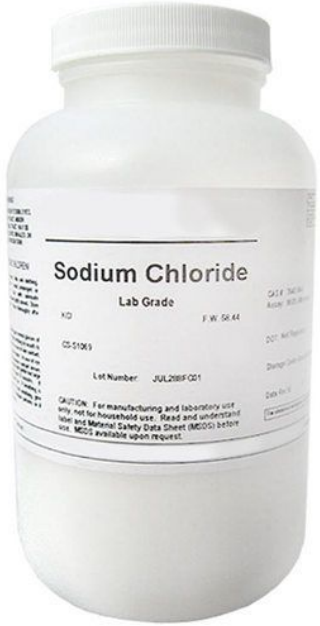




Test tubes containing:
Bradford reagent alone.
(λmax) of the dye 465nm

Test tubes containing:
Bradford reagent with protein added.
(λmax) is shifted to 595nm.





Coomassie Brilliant Blue (470 nm)

Coomassie Brilliant Blue + Protein (595 nm)

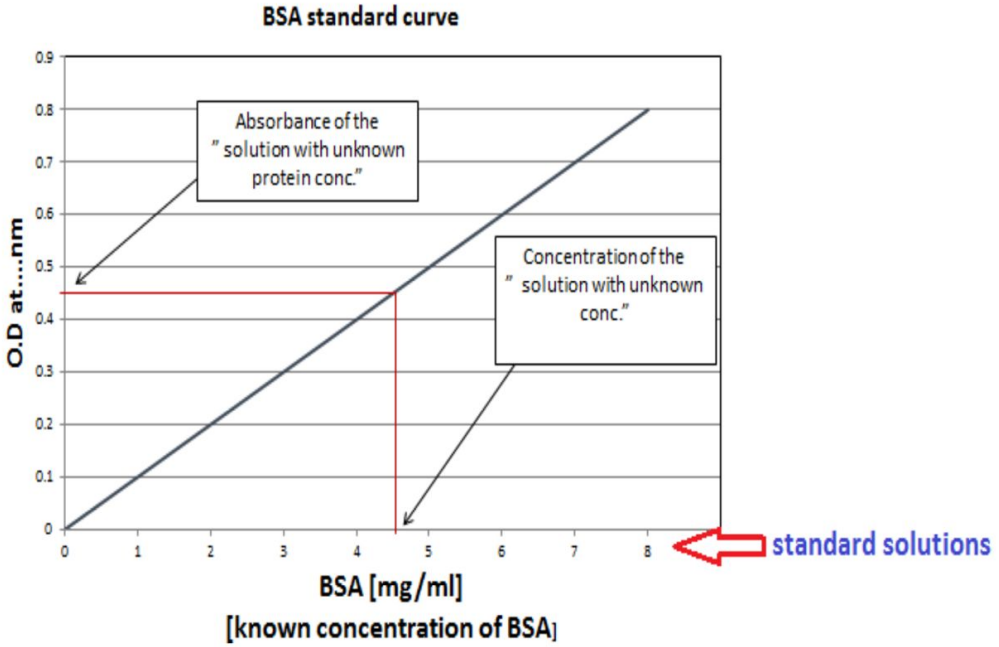
BSA - Bovine serum albumin (BSA) is a blood protein derived from cows. BSA doesn't affect reactions and is low cost, since large amounts can be purified from bovine blood.

When the Bradford reagent (acidified Coomassie Brilliant Blue G-250) binds to proteins, the dye undergoes a color change in the visible spectrum, with the absorbance maximum moving from 470 to 595 nm.

NaCl – sodium chloride - The pH value of the aqueous solution of NaCl is 7 because strong acid and a strong base will neutralize each other effects and a neutral solution forms.

Standard curve

- Standard curve is defined as a graph of known protein concentration of BSA is measured and plotted against absorbance.
- The linear correlation of the protein concentration and absorbance can then be used to determine protein concentration from unknown samples.
- If you graph absorbance versus concentration for a series of known solutions, **standard curve**, which fits to your points can be used to figure out the concentrations of an unknown solution.
- Absorbance, the dependent variable, is placed on the y-axis (the vertical axis).
- Concentration, the independent variable (because it was set by you when setting up the experiment), is graphed on the x-axis.
- When you measure the absorbance of an unknown sample, find that y-value on the standard curve.
- Then trace downward to see which concentration matches up to it.



Protocol & Details

Part A: Standard Curve

Part B: Dilutions and determining the concentration of the unknown

Table 1

- $C_{initial} * V_{initial} = C_{final} * V_{final}$

- What is the concentration of BSA Protein Stock Solution?
  - 0.5mg/ml -> convert to ug/ml!
  - This is your C_1
- We will be solving for V_1
  - This tells us how many ul of BSA protein stock to add to each tube
- C_2 = Final Protein Concentration (given to you in table 1 as ug/ml)
- V_2 = total volume of the reaction = 1ml total

- Use this info to calculate V1
- Then determine how much NaCl to add to bring the volume of the BSA stock + the Bradford reagent to 1000ul

Creating a Standard Curve for Protein Detection

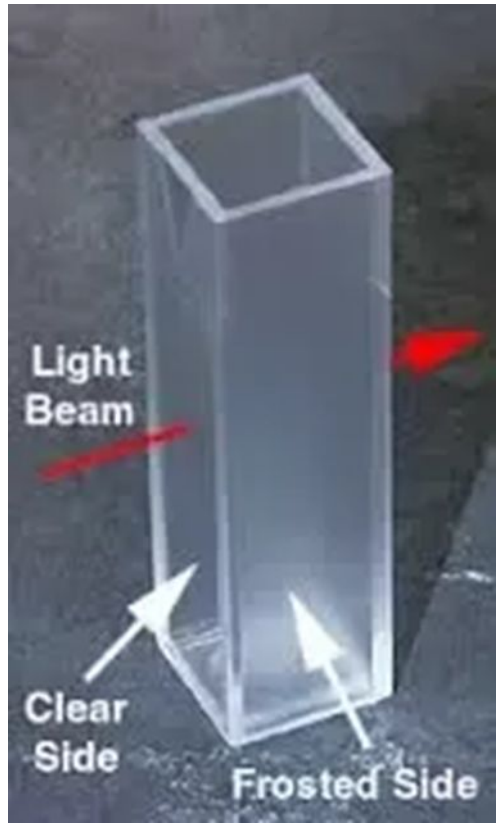
Experiment 2 Standard Curve

Table 1

Tube	Final Protein Concentration	µL of BSA protein stock (0.5 mg/mL) needed	Bradford Reagent	µL 0.15M NaCl for 1.0 mL final volume
8	50 µg/mL	100	900 µL	0
7	25 µg/mL	50	900 µL	50
6	15 µg/mL	30	900 µL	70
5	10 µg/mL	20	900 µL	80
4	7.5 µg/mL	15	900 µL	85
3	5.0 µg/mL	10	900 µL	90
2	2.5 µg/mL	5	900 µL	95
1	0 µg/mL	0	900 µL	100

Protocol Highlights/Tips-Bradford Assay

- Make sure samples are mixed thoroughly
- Touch the cuvettes only at the frosted side
- Make sure the cuvettes are oriented properly when placing them into the spectrophotometer
- Ensure that the samples and controls have incubated 5 minutes after mixing with the Bradford reagent before analysis
- Compare unknown samples to controls by eye before using the spectrophotometer



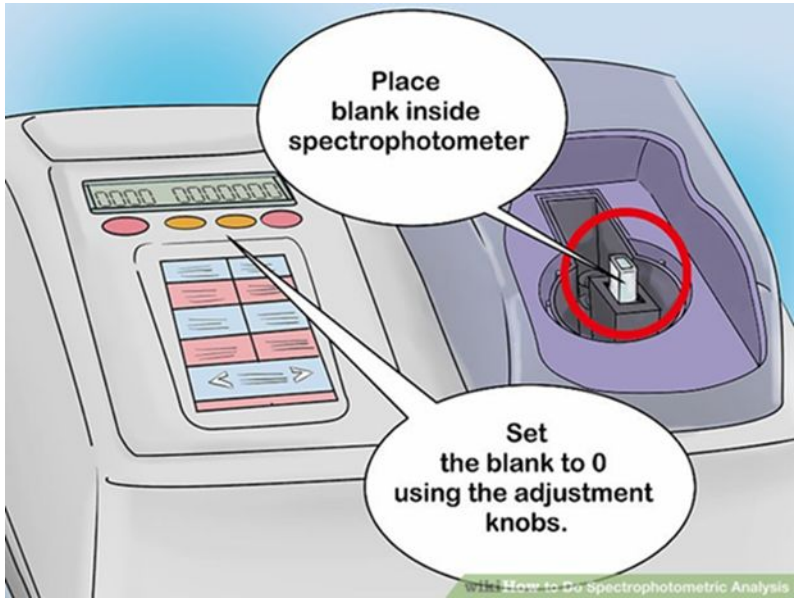
- Compare the samples by eye to the standards
- Spectrophotometers can be used to take absorbance readings at 595 nm
- Graph the controls and use to estimate unknown absorbance

The cuvette on the left is a control with no protein, and the cuvette to the right contain increasing amount of protein.

Protein absent – reddish brown

Protein present – blue

Amount of blue is proportional to protein concentration



The blank is used to calibrate (or zero) the spectrophotometer before measuring the absorbance of the standards and unknown sample.
The standards and unknown sample contain proteins.
Blank has everything except the protein (tube 1/table1).

Protocol Highlights/tips -Standard curve

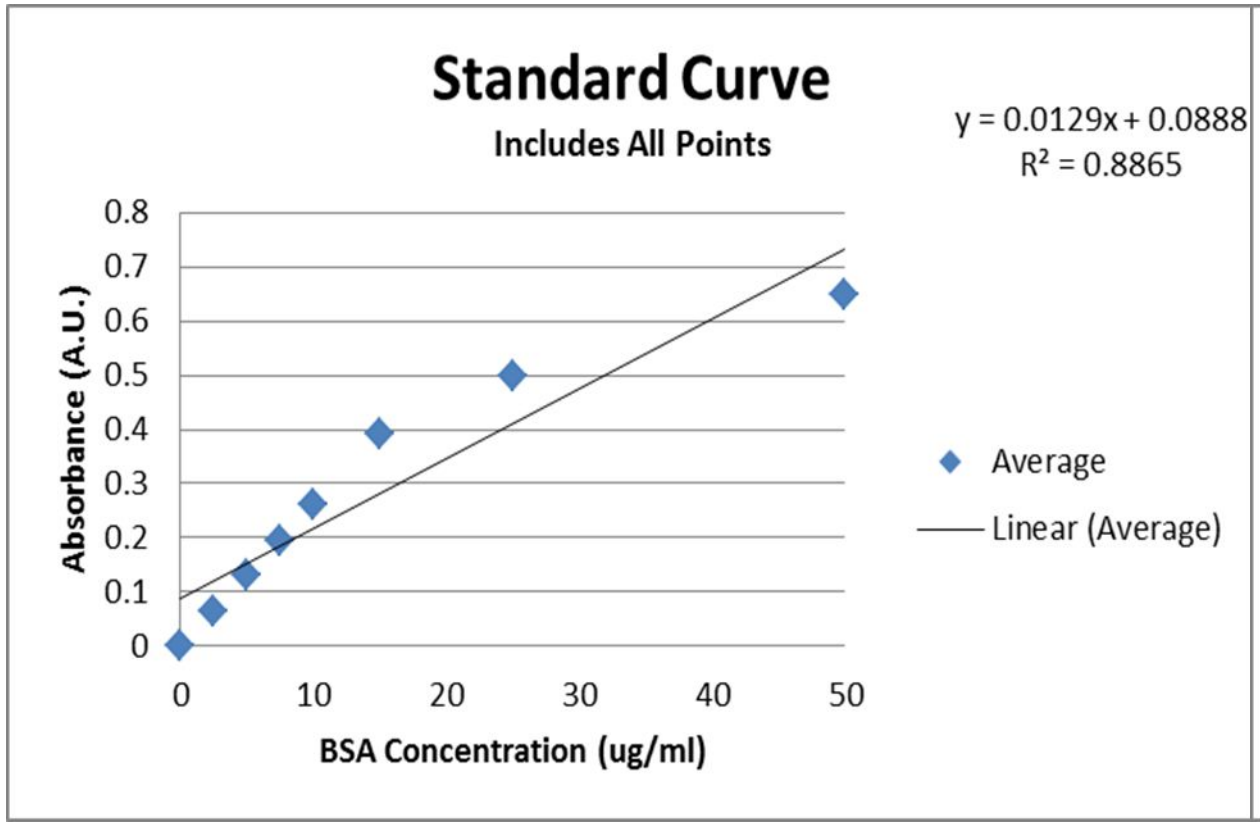
- Notice that as concentration increases, absorbance increases as well.
- While you can estimate concentration of an unknown from just looking at the graph, a more accurate way to determine concentration to use the equation of the line which fits to your data points.
- This equation is given in the y-intercept form: y=mx+b

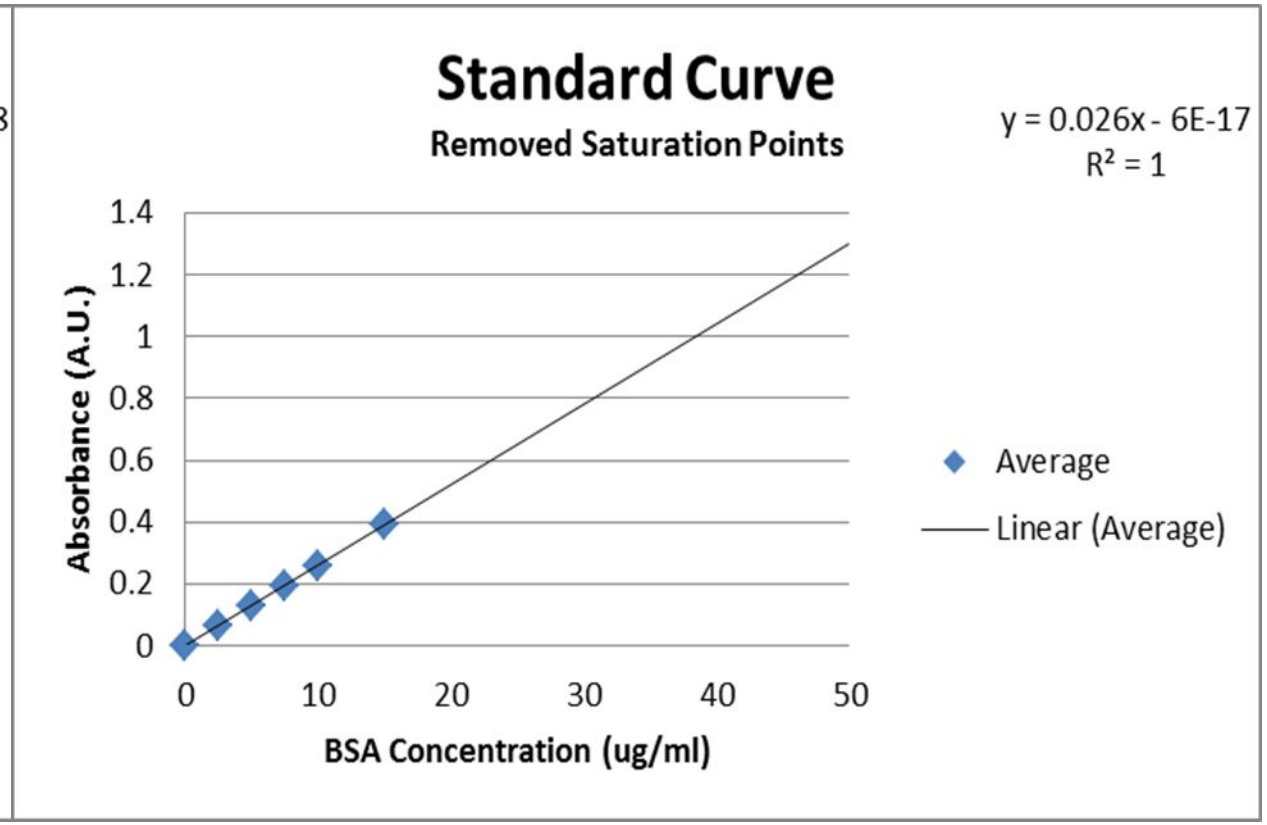
  a = absorbance

  m = slope of the line

  x = concentration

  b = y-intercept (where the line touches the y-axis).
- The slope and the y-intercept are provided to you when the computer fits a line to your standard curve data.
- The absorbance (y) is what you measure from your unknown.
- So, all you must do is pop those three numbers into the equation and solve for x (concentration).





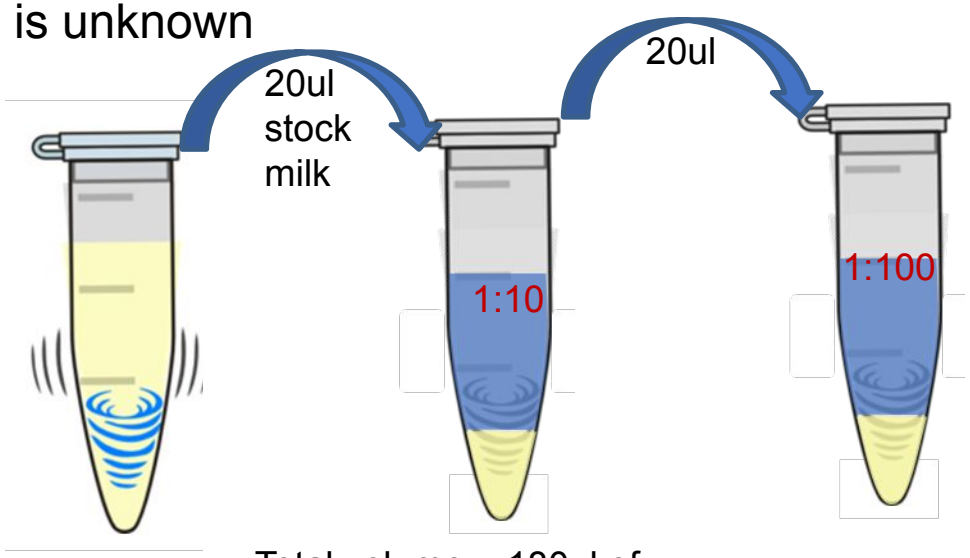
the regression coefficient (R^2) to statistically test how well the data fit the linear equation. The closer this number is to 1.0, the better the data fits the regression line

Table 2	Test #1
Final concentration (ug/mL)	Absorbance (595nm)
0	Your absorbance here
2.5	
5	
7.5	
10	
15	
25	
50	

There is a video in canvas modules labeled “Lab Data analysis” that walks you through how to do this!

Part B: Protein Concentration in milk

Sample of 2% milk
The concentration of protein is unknown



Total volume = 180ul of water + 20ul stock milk = 200ul

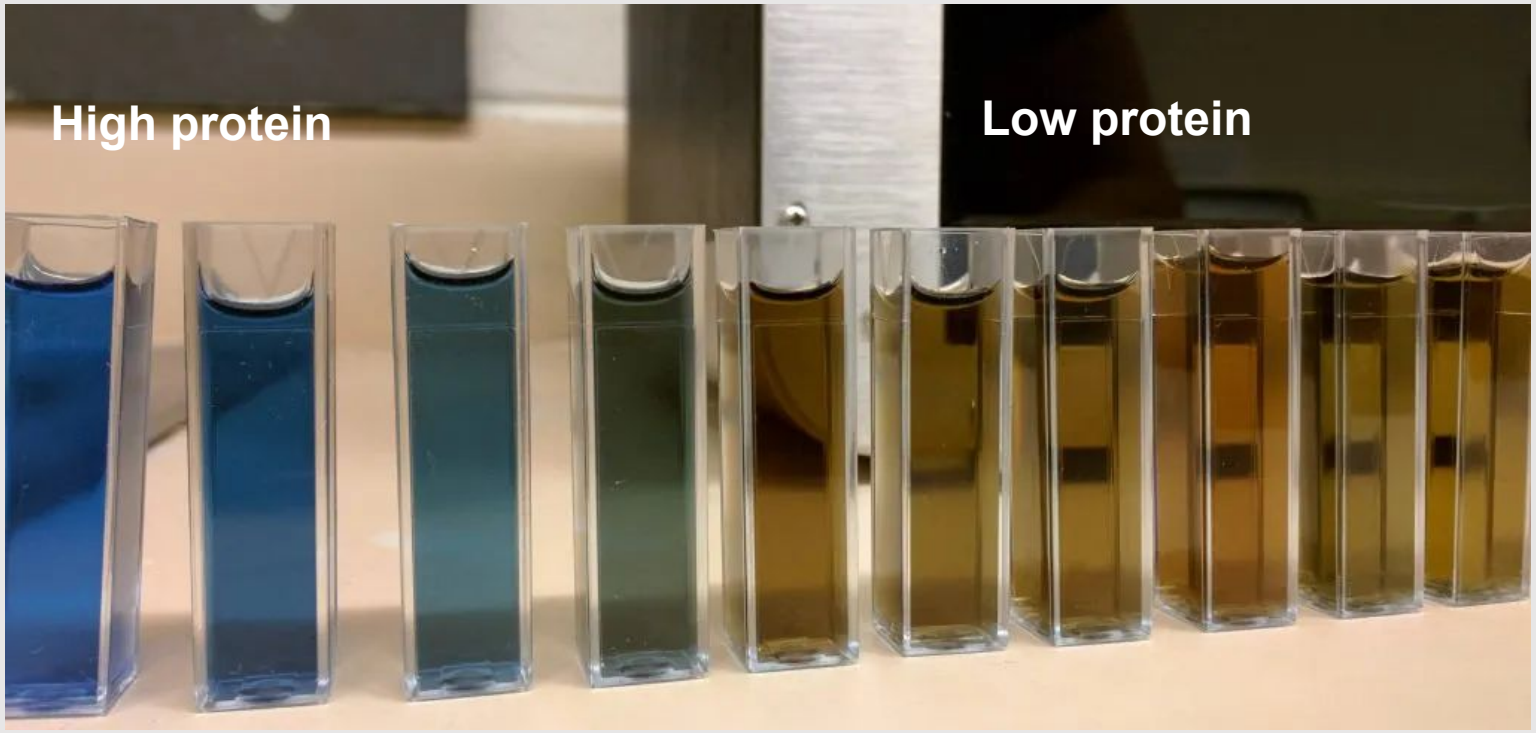
Total volume = 180ul of water + 20ul (1:10) milk = 200ul

… until you get to 1:1000 dilution

Sample 1 1:10 dilution	Sample 2 1:100 dilution	Sample 3 1:1000 dilution

Perform Bradford Assay on Milk Dilutions

- Set up 3 microcentrifuge tubes labeled 1-3
  - Add 900ul of Bradford assay, 90ul of NaCl, then add 10 ul of your diluted milk
    - tube 1 will get 10 ul of the 1:10
    - tube 2 will get the 1:100
    - tube 3 will get the 1:1000
- Compare the color of these reactions to your standard curve
- Select the dilution of milk that is in the middle of the color range



Fill out table 3

- Label 4 new cuvettes 1-4
- Calculate how much NaCl to add to each tube to bring the volume up to 1ml
  - (ex; tube 1: 5ul+900ul+ x= 1000ul where is is the ul of salt that needs to be added)
- Fill your cuvettes: Bradford reagent, NaCl, specified volume of diluted milk (5, 10, 20 or 40 ul)
- Measure this absorbance at 595nm and record

HW assignments

Table 3
My Milk Type = _____ The Dilution of milk I used was: _____

Tube	Unknown Volume V1	Bradford Reagent	0.15M NaCl for 1.0 mL final V2 is 1mL	A-595nm	Concentration Measured by Spec. C2	Concentration of original milk C1
1	5 µL	900 µL	95	0.25	40	
2	10 µL	900 µL	90	0.3	48	
3	20 µL	900 µL	80	0.35	57	
4	40 µL	900 µL	60	0.42	69	

TABLE 3:
To calculate concentration measured by spec:
y= mx + b Where, y = Absorbance, m= slope, x= C2 and b= intercept
y= mx+b Using equation from the graph above y= 0.020x - 0.006 Hence **y = 0.020x - 0.006 (assume 0.006 = 0)**

For example,
y= Absorbance and the absorbance value from the tube 1 of table 3 above = 0.10 **(Note that your own absorbance may be different from this)**
Therefore, 0.10 = 0.020x X=0.10/0.020 X (C2) = **5ug/ml**
(this was the unit used to plot your table 2 graph, so keep it at that)
Now that you have C2.
Using C1V1 = C2V2, you find C1 (Concentration of original milk)

To calculate concentration of unknown volume:
Use C1V1 = C2V2
Where C2 = 5ug/ml, V2= 2ml and V1 for tube 1 = 5uL
C1 x 5uL = 5ug/ml x 2 ml = 10 ug (both ml cross each other out)
C1 =10ug / 5uL C1 =2ug/uL

Then consider the dilution factor you used for your milk:
If you used 1:100 in lab, then multiply C1 by 100,

For example, 2ug/uL x 100 = **200ug/uL**
(This is the unit derived from this calculation, so keep it)