

Determination of Protein Concentration & Use of the Spectrophotometer

In this lab you will review how to use the spectrophotometer. With a spectrophotometer, one can measure how much light of a particular wavelength (color) is absorbed by a solution. The light is absorbed by a particular molecule of interest in the solution. Frequently, that molecule is produced or used up in a chemical reaction proceeds. When we measure these changes, we measure the rate of a reaction as it occurs.

Certain dyes interact with virtually all proteins to yield a protein-dye conjugate that absorbs light differently than the dye alone (there is a significant increase in the light absorption). This change in absorption can be quantified with a spectrophotometer and is the basis of an indirect measure of the amount of protein in a sample. In this procedure, protein containing solutions will be exposed to a dye molecule called Coomassie Blue G-250. The interaction of Coomassie Blue G-250 with protein molecules produces an intensely “blue” complex.

We use special **cuvettes** to hold samples of interest in the “spectrophotometer”. Cuvettes are made of thin, uniform glass or plastic that is highly transparent to light at the wavelengths used. (Some wavelengths such as those in the ultraviolet region (200 to 300 nm) require cuvettes made of other materials such as quartz). Since cuvettes can be very expensive, all cuvettes should be handled very carefully so they are not scratched or broken. Do not put anything sharp or hard into a cuvette. When you use them, always position them such that the light passes through the optical windows and not the sides of the cuvette intended for your fingers. Rinse your cuvettes with distilled water immediately after use to prevent materials from drying onto the inner surfaces.

To use the spectrophotometer, follow the detailed instructions found at: <http://bio.winona.edu/berg/307s06/Labs/documents/Genesys6.doc>. Solutions that are visibly “blue” (such as the solutions containing Coomassie Blue G-250 and dissolved proteins) absorb red light, so you should set the spectrophotometer to provide a measuring beam of red light. Red light has wavelengths ranging from about 600 to about 700 nm. For the maximum sensitivity, a wavelength at the absorption maximum is normally chosen. Red solutions absorb blue light. Blue light has shorter wavelengths (around 400 to 450 nm) than red light. So, to measure a red solution it is necessary to set the spectrophotometer to produce a blue measuring beam. Usually, each lab will tell you what wavelength to use as your measuring beam. However, it is always a good idea to check the absorption maximum for the colored material in question so that you can be confident that you are using the absorption maximum. For measuring protein concentrations in the presence of Coomassie Blue dye we will use a wavelength of 620 nm. Remember, the more colored compound that is present, the greater the absorbance of light. There is more information on the spectrophotometer in the Appendix. Use the step-by step instructions in the Appendix for reference in the future.

Standard Curve for Protein Concentration Determination

One way to determine the protein concentration of a given protein containing solution that has unknown protein concentration is to **compare the unknown solution with a set of protein solutions of known concentration**. The absorbances associated with a set of protein solutions of known concentrations is called a **protein standard curve**. The first step in the determination of the concentration of the unknown solution is to prepare a standard curve using known concentrations of protein. A protein that is frequently used for standard curves is Bovine Serum Albumin (BSA). BSA is desirable because it is commercially available as a dried powder that is easily weighed with a laboratory balance. BSA readily dissolves in water and forms a colorless solutions. Dissolved

BSA reacts with Coomassie Blue G-250 to form a blue colored addition product. In this lab you will: A) mix known concentrations of BSA dissolved in buffer with Coomassie Blue G-250, B) measure the absorbance of the resulting blue colored solution and C) plot the measured absorbance of the protein-dye conjugate versus the known concentration of BSA in that sample. The **resulting graph will be the protein standard curve** that you will then use to determine the amount of protein in your samples of unknown protein concentration.

NOTE: You will be provided with a protein solution containing BSA at a concentration of 100 µg/ml. This is known protein concentration. You will do serial dilutions of your known protein concentration to make other proteins solutions of known concentration.

1. Make sure that the spectrophotometer has been turned on and is fully warmed up.
2. Label two sets of 13 x 100 glass tubes #1-6a and # 1-6b. (You will do each of the following steps in duplicate).
3. Your BSA working solution is 100 µg/mL. You must now calculate what volume of the BSA working solution you must add to the tubes such that the two sets of six tubes will contain 0, 10, 20, 40, 70, or 100 µg protein respectively. If in doubt, check with the instructor. Write your volumes in the table below.
4. To each tube add the appropriate amount of distilled water to bring the final volume up to 1 mL. All tubes must have the same final volume. Write your volumes in the table below.
5. Add 1 mL of Coomassie Blue G-250 Dye Solution to each tube. Mix by inverting (use parafilm over the top of the test tube), swirling or gently vortexing with a vortex mixer. (Remember, frothing denatures proteins; denatured proteins precipitate, and precipitated proteins are not accurately measured. Only soluble protein is measured).
6. Tube #1 contains no protein so it is considered the "blank". Pour the contents of tube #1 into a cuvette. Place this cuvette in the cuvette holder labeled "B".
7. Sequentially place the contents of the other tubes into cuvettes and place them sequentially into the cuvette holders 1, 2, 3, 4 and 5 on the rotating turret. Close the lid.
8. Allow the color to develop for 10 minutes. While the color is developing, zero the instrument using the cell in the "B" position as described at:
<http://bio.winona.edu/berg/307s06/Labs/documents/Genesys6.doc>
9. Ten (10) minutes after adding the protein dye solution, measure and record (see the Table below) the absorbance of the cuvettes in positions 1, 2, 3, 4 and 5 at 620 nm. [Note: the color will continue to develop and get darker with time. The standard curve will only be useful when compared to samples of unknown protein concentration that have been similarly exposed to the protein dye solution for 10 minutes.]
10. To create a standard curve, plot the net absorbance or A₆₂₀ of each sample on the Y-axis versus the µg protein (BSA)/mL on the X-axis. Look at the graph and find the linear region. You will use the linear range to determine the protein concentration in your unknown sample.

Tube Number	µg BSA to be added	Volume BSA solution to be added	Volume of water to be added	Concentration of BSA in ug/mL	Average Absorbance at 620 nm
1	0				
2	10				
3	20				
4	40				
5	60				
6	80				
7	100				

Determination of Protein Concentration in a Sample of Unknown Protein Concentration

Preparation of samples: Since you don't know the protein concentration of your sample (it might be greater than or less than the concentrations present in your standard curve) it is best to do some dilutions of your unknown to increase the likelihood that you will be able to produce a sample with an A_{620} that falls somewhere on your standard curve. Dilute your fractions with water indicated in the table below.

1. For a 1/10 dilution, pipette 100 μ L of unknown protein solution into a clean cuvette; add 900 μ L distilled water and invert to mix. For a 1/20 dilution, add 50 μ L of unknown protein solution + 950 μ L distilled water. For a 1/100 dilution, add 10 μ L of unknown protein solution + 990 μ L distilled water. Using these three dilutions, continue with the following instructions:
2. Add 1 mL of Protein Dye Solution to the 1 mL of diluted protein. Make a Blank by mixing 1 mL of Protein Dye Solution with 1 mL of distilled water. At this point you should have a Blank and 3 solutions of unknown protein concentration.
3. Determine the A_{620} of all three unknown protein solutions
4. If any of the absorbances fall off of the range of your protein standard curve, then the absorbance reading is invalid. If all of your absorbances fall off the range of your protein standard curve then you should repeat the measurement using the greater dilution of unknown protein solution.
5. If the absorbance reading is in the linear range of the curve, and above the lowest detectable amount, record this value and use it to determine the protein concentration of that fraction.
6. Using the BSA standard curve, determine the protein concentration of each fraction in μ g/mL
7. When finished, rinse all tubes thoroughly in warm running tap water and then submerge them in pan of water for later washing.
8. Cuvettes should be carefully rinsed with warm running tap water and the inside surfaces of the cuvettes should be gently rubbed with wet cotton swabs to remove any adherent Coomassie stain. The cuvettes should be rinsed one final time in RO water and turned upside down to dry on a piece of absorbent material or paper towel.

	Dilution	Abs 620 nm	[Protein] from standard curve	[Protein] of the original sample
Unknown 1/10	1/10			
Unknown 1/20	1/20			
Unknown 1/100	1/100			

Protein (BSA) Standard Solution: 0.1 mg BSA/mL (100 μ g BSA/mL)

Protein Dye Solution: 600 mg Coomassie Brilliant Blue G in 50 mL 95% ethanol plus 750 mL 0.4 M perchloric acid. Dilute the combined solution to 1 L. Filter prior to use.

Perchloric Acid (0.4 M): Dilute 34.5 mL concentrated perchloric acid to 1 L with distilled water