

Experiment: Bradford Protein Determination (Skim Milk)

The Bradford protein assay is a rapid, simple protein concentration determination method in solutions. The structure of the dye is shown in Figure 1. It is based on the equilibrium between three forms of Coomassie Blue G dye (Scheme 1).

Under strongly acid conditions, the dye is most stable as a doubly protonated pale red form ($\lambda_{\text{max}} = 470 \text{ nm}$). The anionic form of the dye may noncovalently bind to proteins primarily through reacting with the basic and aromatic amino acid residues of proteins. This bound form of the dye is most stable as an unprotonated blue form ($\lambda_{\text{max}} = 595 \text{ nm}$). The blue protein-dye form can be detected at 595 nm in the assay using a spectrophotometer. By selecting an appropriate ratio of dye volume to protein sample concentration, Beer's law may be applied for accurate quantitation of protein, as long as the protein concentrations are chosen to be within the linear range. This is due to the extinction coefficient, ϵ , of the protein-dye complex solution being constant over a 10-fold concentration range.

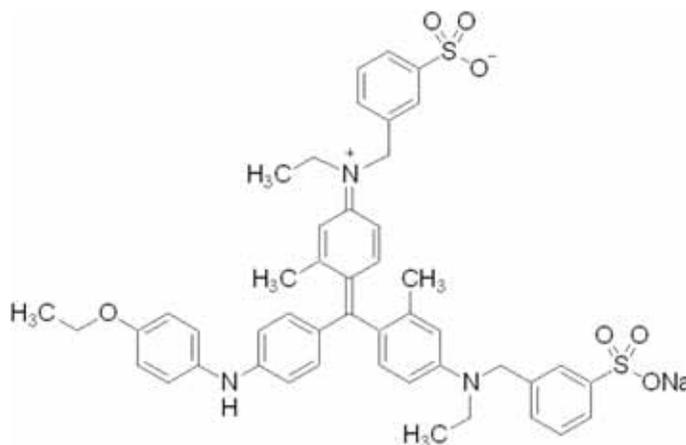
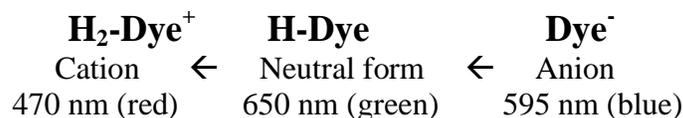


Figure 1. The structure of Coomassie Brilliant Blue G-250.

Scheme 1



The Bradford assay is not susceptible to interference by a wide variety of chemicals present in samples. Some non-protein compounds, however, such as detergents and basic protein buffers may interfere with the Bradford assay by shifting the equilibrium of the dye and altering the relative concentrations among the three colored forms.

Standard protein samples need to be prepared to give absorbance values within the linear range of the assay and unknown protein samples to be tested must be approximately within the range of the standards minimize variations in absorbance values obtained using the Bradford procedure.

PROCEDURE:

Note: Do not dry the dye or any protein samples in the oven.

Preparation of Dye:

Preparation of the dye can be requested from the Graduate Assistant (GA). To prepare enough for 6 students, measure 0.05 g of Coomassie brilliant blue G-250 (Dye) on weighing paper. Place the dye in a 100 mL beaker. In the fumehood, add 25 mL of 95% ethanol and 50 mL of 85% phosphoric acid. After the dye completely dissolved, completely transfer the dye solution to a 500-mL volumetric flask and add distilled water to the mark on the volumetric flask.

Students can obtain 80 mLs of the dye from the GA. Before use, filter the dye through a Whatman #1 filter paper. (Note: Keep the dye in an acidic environment).

Preparation of Standard Protein Solutions:

Weigh accurately 0.02g Bovine Serum Albumin (**BSA**) on a weighing paper and dissolve it into in about 10 mL water in a clean beaker. Transfer the resulting solution into a 100-volumetric flask and make it up to volume with distilled water. Calculate the concentration of the stock BSA in ppm (show that it is ~200 ppm protein).

Prepare and label ten (10) 1.5-mL Eppendorf centrifuge tubes for working standard samples and unknowns. Six of the tubes are for the standard curve and two tubes for each unknown. Prepare the six working standard solutions using the stock solution. Pipette 5, 12.5, 25, 37.5, 50, 62.5, and 75 μ L stock BSA solution into 1.5 mL Eppendorf centrifuge tubes and pipette distilled water to bring the final volume to 100 μ L, respectively. Add 1.4 mL of the filtered dye to each tube and mix well and set the samples aside on the bench for ~10 min to allow them to equilibrate.

Template table for standard sample preparation:

Sample Conc. (ppm)	Stock BSA (μ L)	Distilled water (μ L)	Total Vol. (μ L)
10	5.0	95.0	100.0
25	12.5	87.5	100
50	25.0	75.0	100
75	37.5	62.5	100
100	50.0	50.0	100
125	62.5	37.5	100
150	75.0	25.0	100

Preparation of the Unknown Solutions:

Ask your GA for 100 μ L of unknown protein solution and 100 μ L fat-free skim milk. Keep the samples in ice and add 0.0516 g ammonium sulfate (NH_4SO_4) to each unknown solution (final solution will be 80% saturated in relation to ammonium sulfate). Mix well, if possible, vortex the samples to make sure ammonium sulfate completely dissolves into the solutions. (Increasing the ionic strength causes the protein molecules in solution to precipitate without irreversibly denaturing the proteins. It can be redissolved in the later after isolation.)

Centrifuge the samples using an Eppendorf Centrifuge. Note: put the two samples in the opposite position of the rotor in order to balance. It is important to ascertain that the tubes are balanced to ensure that the centrifuge will not be damaged. Centrifuge at 12,500 rpm for 10 min.

The pellet will appear at the side of the tube. Decant the supernatant of the sample but keep the pellet. Resuspend the pellet into 100-500 μL distilled water, respectively, and completely transfer it to a 100 mL volumetric flask. Then take 100 μL of sample from the flask into a 1.5 mL centrifuge tube and add 1.4 mL of the filtered dye to each tube. Mix well and set the samples aside on the bench for approximately 10 min to allow them to equilibrate. The Bradford assay is stable for at least an hour after mixture.

Refer to your textbook or notes for a more detailed discussion of Beer's Law.

SPECTROPHOTOMETRIC DETERMINATION (refer to your textbook for appropriate theoretical discussion):

It is convenient to express the concentration in terms of parts per million (ppm) instead of using molar concentrations since the initial weights and the final volumes of the unknowns and standards are equal to each other. It takes about 1-1.5 mL of solution in the sample cell to obtain a reading. Follow the operating procedure below.

Note: do all determinations in duplicate or triplicate. Fill the reference cell with distilled water and the sample cell with one of the standard BSA solutions. Turn on the spectrophotometer and wait ~5 minutes to warm it up. Adjust the spectrophotometer to a wavelength of 595 nm, and blank the spectrophotometer using the reference cell. Take readings of the samples at 595 nm. (To better understand the behavior of the dye in relation to the protein concentration, you may also take readings of the samples at 470 nm after blanking with the reference cell.)

Be sure to use the same sample cell for all your calibrations and measurements. Record your results in the same table format as on the attached sheet. Since the same sample cell is used several times, it is important to rinse the sample cell quantitatively with the next unknown sample solution to be measured. Determine if Beer's Law is observed by plotting (see sample graph attached in the informal lab sheet) Absorbance at 595 nm (A , y-axis) vs. concentration (ppm, x-axis). The standard (calibration) curve is obtained by drawing the best straight line that most closely approximates the data points. Use this curve to determine the concentrations of the unknowns corresponding to the absorbance of the unknown solutions. Both of these plots are to be included with your informal report.

It is possible to determine the protein concentration of the unknown algebraically by using the Beer's Law equation and taking ratios. For example, $A_{\text{unk}} = \epsilon c_{\text{unk}} b$ and $A_{\text{std}} = \epsilon c_{\text{std}} b$ or, $A_{\text{unk}}/A_{\text{std}} = c_{\text{unk}}/c_{\text{std}}$. where $c_{\text{unk}} = (\text{ppm})_{\text{unk}} (\text{mass})_{\text{unk}}/V_{\text{unk}}$ and $c_{\text{std}} = (\text{ppm})_{\text{std}} (\text{mass})_{\text{std}}/V_{\text{std}}$. In this experiment, the volumes are equal: $V_{\text{std}} = V_{\text{unk}}$. Or, one can, calculate the molar absorptivity, ϵ , of the dye-protein complex from the standard sample and apply this to the unknown sample using Beer's Law.

Because the algebraic methods of calculation outlined above presume that the data lie on the standard curve, it may be more prone to error than the graphical method using the standard curve. As such, the informal report should be based on the graphical method for greater accuracy.

FURTHER READING:

1. M. M. Bradford, "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principles of Protein-Dye Binding", *Analytical Biochemistry* (1961) 72, 248-254.
2. S. J. Compton and C. G. Jones, "Mechanism of Dye Response and interference in the Bradford Protein Assay", *Analytical Biochemistry* (1985) 151, 369-374.

***Operating procedures for the spectrophotometer:**

Turn on the spectrophotometer using the on/off switch at the back of the device and let it warm up for about 10 minutes. Have 2 identical cuvettes – one a reference cell (to be filled with the "blank" solution, which in this case is water), and the other a sample cell (to be filled with the absorbing solutions). Have all your solutions (the 2 undiluted unknown solutions, the standard #1 -both undiluted and diluted, and standard #2 which acts as a back-up) ready when measurements are made.

It is important to make all the measurements in a single sitting to minimize drift in the data. It is necessary to "zero" the absorbance first whenever the wavelength is changed. To zero the absorbance, set the wavelength by scrolling the wavelength up or down using the "nm" or "nm" buttons. Insert the reference cell (with the blank solution) into the cuvette holder making sure it is properly aligned to maximize the pathlength through the solution. Close the cover and press the "O Abs" button and wait for the digital read out to adjust to zero. The sample cell can now be inserted in place of the reference cell. The digital readout is the absorbance data for the sample solution.

Note: Discard all waste in appropriate bottles as instructed. If you have any questions, please see your instructor or teaching assistant before disposing of waste.

Informal report:

Date submitted: _____

Experiment: Bradford Protein Determination Time submitted: _____

Name: _____

Values obtained:

(1) _____ ppm

Locker # _____

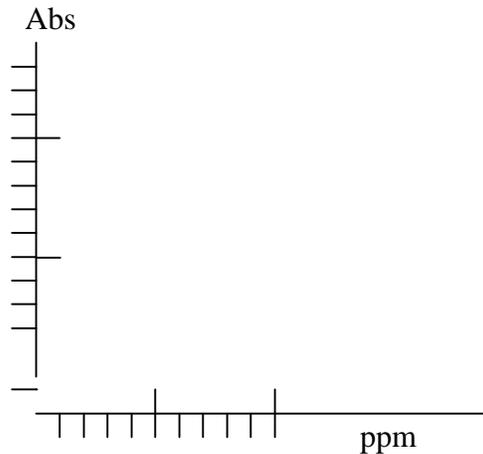
(2) _____ ppm

Recovery = (1) _____ %

(2) _____ %

Data for calibration curve

Solution (expressed as ppm)	Absorbance
10	
25	
50	
75	
100	
125	
150	
unknown #1	
unknown #2 (milk)	



Note: Draw the calibration curve in your laboratory notebook and attach a copy with your lab report as you turn it in to be graded.

Questions:

1. Name two more methods to determined protein concentration and give a brief description on these methods.
2. List two main advantages and disadvantages of Bradford Assay.