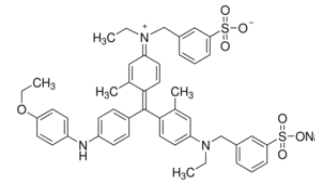




**Theory and Introduction: Protein Assay** –There are many reasons to conduct a protein assay. During a purification of a protein, you need to know how pure your sample is by determining the amount of enzymatic activity vs. protein concentration. When comparing samples on an SDS-PAGE gel or using antibody for a western blot, you need to add the same amount of protein from various preparations to the true results. Most protein assays take advantage of a reaction between a reagent dye and the protein of interest that will shift or increase the absorbance of a particular wavelength. There are only a few regularly used methods to determine protein concentration. The Bradford assay is described below. The Lowery/Bicinchoninic acid assay (BCA), utilizes a copper-protein chelate where the absorbance of a reduced copper solution is measured to detect protein. The uv absorbance of a protein, contributed primarily by three residues, Phe, Tyr and Trp are used in what is called the Christian Warburg method or using the extinction coefficient. However, the individual composition varies from protein to protein. Some proteins contain few if any Trp residues, while Tyr and Phe can change several fold from protein to protein.



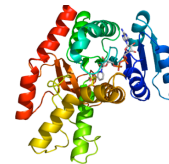
Structure of CBB G-250

**Bradford reagent** The Bradford protein assay is a colorimetric protein assay originally described by Marion Bradford (*Anal Biochem* **72**:248-54, 1976) which uses a disulfonated triphenylmethane compound called, Coomassie Brilliant Blue G-250 (CBB G-250). Without protein in acidic solution the dye red-brown. Under acidic conditions the protein binds to mostly basic amino acids such as arginine, lysine and other amino acids including histadine. Under these conditions, dye-amino acid binding results in a shift dye pKa of the dye causing the color to change from green/brown tint to blue. The protein-bound dye is measured at 595 nm. The more protein, the more intense the blue color. Amino acid composition as well as post-translational modification of proteins can lead to variation of dye intensity. Care must also be taken that the dye is not the limiting reagent in the assay as this will result in a non-linear standard curve. Bradford dye is easy to use, is fast and sensitive, but several compounds can interfere with the assay (conduct a search or look on the biorad website for Bradford reagent interfering compounds).

**Standard curve:** A protein assay consists of two main components; the standard curve and the unknowns. How does this absorbance relate to the actual protein concentration? To know determine the actual concentration of a protein a standard curve is required. A standard curve is a plot of absorbance vs. a varying amount of some known protein that you know the concentration. Then you will do the same in parallel with unknown protein sample (in this case the fractions or pools of your purification). A common method to prepare a standard curve is to prepare various known protein concentrations as standards. As long as the volume of the standard samples and the unknown samples are the same the final concentration of the unknown is directly calculated from the least squares line of the standard curve. Of course you have to correct for any dilution of your sample.

Depending on the method, proteins generally vary in the reaction with the color dye in the different protein assays. Therefore, it is important to denote what the specific control or reference protein was used to make the standard curve. Two common proteins used for standard curves are bovine serum albumin (BSA) and an immunoglobulin (IgG). These two proteins have a different amino acid composition, which leads to a different standard curve and a slight difference in the final determination of the unknown protein concentration. Because the color development is dependent on the amino acid composition of the protein and the presence of a prosthetic group (especially carbohydrate) also influence the protein assay; a purified sample the protein being assayed or a closely related protein is a preferred standard.

**Be linear:** Once you have performed the assay, a standard curve is generated and the results graphed. Before using the standard curve you've created, you must be certain that the absorbance is a linear function of concentration that holds within the limits of the Beer-Lambert Law. Beer's law does not hold at high concentrations. A common reason for a lack of linearity, is the depletion of one of the reagents necessary for color production. Another reason for error is that the absorbance is too high. Remember that absorbance is 1/transmittance (the spec is really reading how much light goes through the sample). Therefore, a high absorbance means very little light is making it through the sample. When the absorbance is very high (1.2-1.5 for plate assays and 1.7 – 2.5 for spectrophotometers) you should look close at your data.



If the standard curve is leveling off, then you should not use the points with the higher absorbance. Thus readings should always be taken in the region where all reagents are in excess where the curve of abs vs. concentration is linear.

**Sample Preparation:** When determining the protein concentration of an unknown sample, several dilutions should be used to ensure the protein concentration is within the range of the assay. Usually 10 fold dilutions are used to get the unknowns within the standard curve range. While this may seem redundant and a pain in the neck often times your sample may be too diluted to fit within the standard curve. Without the additional dilutions, you will have to start over again. Don't forget that all dilutions must be taken into account in calculating the final concentration of the protein.

Finally, with every assay a "blank" must be included. The blank or the tube without a standard protein is usually made up in the same buffer as in the samples. This way if the buffer has an effect on the protein assay reagent, each sample (and the blank) will have been altered the same. The "blank" is used to set the instrument to the 100% transmittance or 0 absorbance

**Protocol – NOTE: USE THE CLEAR 96 WELL PLATE. FIRST ADD BRADFORD THEN ADD PROTEIN SAMPLES (EITHER STDS OR UNKNOWN) TO THE BRADFORD IN THE SAMPLES.** Calculate out your additions in your lab book in table format.

1- Prepare 0.5 ml of each known BSA protein standards. Freeze unused std for your next assay!

- The stock BSA concentration is 1 mg/ml. Use water to dilute. Adjust if the concentration is different
- Protein standards = 0.0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml.

2- Determine how many wells you will need for your assay.

- Run EACH standard in triplicate **If reading column fractions, n=1 will be fine.**

3- Add 250  $\mu$ l of 1X Bradford reagent in each well.

4 – Add 10  $\mu$ l of EITHER BSA standard OR your unknown sample (fraction) to the Bradford Reagent.

5- Determine the absorbance at 595 nm.

- Turn on the plate reader FIRST then the computer. There is no password, leave blank
- Click on the plate reader **KC4 icon** on the desktop
- Use methods or protocols to find the Bradford assay file and measure your protein.
- Export your data to an excel file and transfer to a USB file for your notebook.

6 – Wash your plate and if needed a rinse with methanol followed by water to remove residual blue dye.

### Calculations –

1. Prepare a graph of the standards (the standard curve) with the dependent variable (mg/ml) on the X axis and the independent variable (abs 595 nm) on the Y axis.
  - Calculate the protein concentration of your knowns using  $CV=CV$ . See #3 below.
  - You do NOT know the protein concentration of your experimental/unknown samples, therefore the unknown samples/fractions/experimental assays can NOT be graphed on your standard curve.
  - Do not "connect the dots" of the standard curve, instead perform a linear regression to your data.
  - Show the regression "r" value in the box of your graph. The closer to 1.0 the number the more in line your data are. 0.9 to 1.0 are reasonable values. *Look at the line, if the last one or two points indicate that the absorbance does NOT increase in a linear fashion with added protein, exclude these values from your curve. You have saturated the assay or are beyond beer's law.*
2. Use the linear regression to calculate the concentration in your unknown samples. If you diluted your sample, don't forget to multiply the concentration by your dilution factor.

3. When doing your calculations, ignore the volume of the Bradford reagent. You need to know the concentration of protein in your original sample NOT the cuvette!