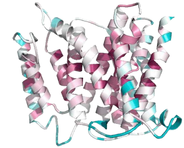
PROVOST & WALLERT RESEARCH

Investigating the Biochemistry & Cellular Physiology of NHE1
EST. 1998

Bradford (BioRad) Protein Assay
1.5 ml Protocol



Theory and Introduction: The determination of protein concentration is frequently required in biochemical work. Several methods are available, each having features that suit it to a particular use. 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 obtain 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. Generally, the more protein in a sample, the higher the absorbance. BUT remember the assay must remain linear and follow Beer's Law! If the absorbance is higher than 1.5 absorbance units (AU), then look very close at your data before using it!

Bradford reagent (we use the reagent prepared by BioRad Protein Assay Solution) uses Coomassie blue G-250. Without protein, the solution is red-brown in its acidic solution. When protein binds, the pKa of the dye shifts causing the dye to become blue. The dye is measured at 595 nm. Bradford dye is easy to use, as well as fast and sensitive, but several compounds can interfere with the assay (*see the pdf file for the BioRad Protein Assay on the class webpage*).

Standard curve: *Learning how to prepare and use a standard curve is an incredibly important skill that all employers and professional schools expect you to know*. Thus you will be conducting several standard curves while doing a protein assay throughout this semester. A protein assay consists of two main components: the standard curve and the unknowns. Bradford and Lowry protein assay reagents results in a change in absorbance when protein is present. 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 concentration of protein. Then you will take absorbance readings 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 that may be needed to achieve an absorbance that falls within your standard curve.

Always run samples in duplicate or triplicate and use the averages in the graph. If a sipper is used for reading samples, it is a good idea to read those samples with less protein content first to reduce error arising from reagent dye carryover in the cuvette as a result of incomplete rinsing.

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 immunoglobin (IgG). These two proteins have different amino acid compositions, which lead 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) which can also influence the protein assay, a purified sample the protein being assayed or a closely related protein is a preferred standard.

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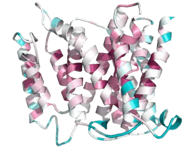

Once you have performed the assay, a standard curve is generated and the results graphed. Before using the standard curve you've generated you must be certain that the absorbance is a linear function of concentration, which holds within the limits of the Beer-Lambert Law. Beer's Law does not hold at high concentrations. A common reason 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 (1.7 – 2.5 depending on the instrument), 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 absorbance 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 (or other body parts), often times your sample may be too concentrated 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.

Location: The 1X Bradford (BioRad Protein Assay) solution is in re-pipettor in the west hood. The concentrated BioRad Protein Assay solution is a 5X solution to be diluted with MilliQ water. The BSA (1 mg/ml) is in microfuge tubes in the freezer. Re-freeze any unused BSA.

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 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 fit your data.
   - Show the regression "r" value in the box of your graph. The closer to 1.0 the number the more linear your data points 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. ONLY use the volume of the sample (30 μl). All tubes have the same amount of reagent and can thus be ignored.

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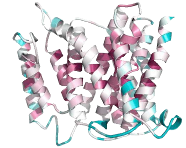

Protocol: You will need to prepare three sets of microfuge tubes. One tube with the buffer used in the experimental unknowns and Bradford is the blank for the spectrophotometer. The second set of tubes is used for your standards and the third set of tubes is the unknown samples (fractions and pooled samples).

1. Determine how many microfuge tubes you will need. You will need one tube for the blank, 14 tubes for the standard curve, and one or two tubes for each of your unknown experimental samples. The fractions need to only be read once. All other samples should be assayed in duplicate or triplicate. *Number each tube.*
2. Add 1.4 ml of 1X Bradford reagent in each microfuge tube. Use the re-pipettor or your 1.0 ml pipet.
3. To the blank tube, add 30 µl of the buffer you samples are in. Use this to blank the spectrophotometer.
4. Place following amounts of BSA protein standard (1 mg/ml) or buffer from your sample into the standard tubes.

Tube	µl BSA	µl Buffer	Calculated BSA Conc (mg/ml)
2	0.0	30.0	
3	0.0	30.0	
4	5.0	25.0	
5	5.0	25.0	
6	10.0	20.0	
7	10.0	20.0	
8	15.0	15.0	
9	15.0	15.0	
10	20.0	10.0	
11	20.0	10.0	
12	25.0	5.0	
13	25.0	5.0	
14	30.0	0.0	
15	30.0	0.0	

5. Add up to 30 µl of unknown (record volume) to each test tube. Add the appropriate volume of H_2O to QS to 30 µl. Briefly vortex and compare to the standards. The unknown should be darker than the color in tubes with 5 µl of BSA, but should be less intense than tubes the top two concentrations of BSA in your standard curve. If your sample does not fit into this range, dilute at least in half and check again.

6. Determine the absorbance at 595 (VIS lamp). Use the sipper or using individual cuvettes. Make sure to check the settings and use the fixed wavelength. Review the instructions for using the spectrophotometer if you have forgotten.