Lab 7 Protein Quantitation & SDS-PAGE

Protein Quantification Background

Biochemical research often required the quantitative measurement of protein concentrations in solutions. Several techniques have been developed. Most have limitations because they either are not sensitive enough or they are based on reactions with specific amino acids in the protein. Since the amino acids vary from protein to protein, no single assay will be suitable for all proteins.

The oldest methods are the Biuret and Lowry assays. In the biuret assay alkaline copper sulfate reacts with peptide bonds to produce a purple complex. The amount of purple formed depends on the amount of protein present. The Lowry assay is one of the more sensitive assays (as low as 5µg). The principle is the same as the biuret assay, but a second reagent (Folin-Ciocalteu - binding aromatic amino acids) is added to increase the amount of blue color developed. This assay is time consuming. And since the amount of tyrosine and tryptophan varies between proteins, different proteins may produce different results.

The most used assay is the **Bradford assay**. This is a rapid and accurate method for the estimation of protein concentration. The assay was originally described by Bradford (1). This technique is simpler, faster (one step reaction), and more sensitive than the Lowry method. It is also subject to less interference by common reagents and non-protein components (such as: sodium, potassium, & carbohydrates).

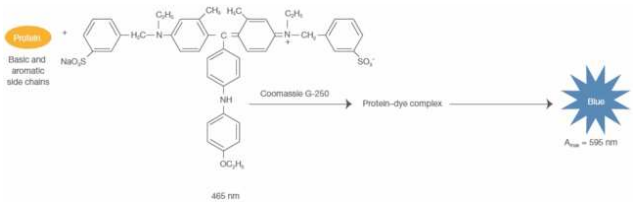
The Bradford assay relies on the binding of the dye Coomassie Blue G250 to protein. The dye forms a strong, noncovalent complex with the protein's carboxyl group by van der Waals force and amino groups through electrostatic interactions. This results in a spectral shift from the reddish-brown form of the dye (absorbance maximum at 465 nm) to the blue form (absorbance maximum at 610 nm). The difference between the two dye forms is greatest at **595 nm**, making it the optimal wavelength to measure. Free amino acids, peptides, and low molecular weight proteins do not produce color with Coomassie dye reagents. In general, the mass of a peptide or protein should be at least 3,000 Da for quantification with this reagent.

Start by making a serial dilution of a known amount of protein. Measure the absorbance at 545 nm and create a standard linear curve (absorbance versus protein concentration in _g/mL). Then measure the absorbance of the unknown protein solution. Use the graph (line equation) to determine the protein concentration.

The main disadvantage of Bradford protein assays is their incompatibility with detergents at concentrations routinely used to solubilize membrane proteins. In general, the presence of a surfactant in the sample, even at low concentrations, causes precipitation of the reagent. This limitation can be overcome by the addition of cyclodextrins to the assay mixture. Another disadvantage is that the reagents tend to stain test tubes. The same test tubes cannot be used since the stain would affect the absorbance reading. As a result the assay will be performed in disposable plastic 96 well plates.

A final way to measure protein concentrations is by aromatic amino acids absorbing light in the ultra violet spectrum at 280 nm. This is often used following pressure chromatography. Caution should be observed since other cellular components can absorb strongly around 280 nm. In particular, nucleic acids absorb strongly at 280 nm (λ_{maxc} = 260). If a sample contains up to 20% nucleic acids use:

$$[\text{Protein}]\ (\text{mg/ml}) = 1.55A_{280} - 0.76A_{260}$$

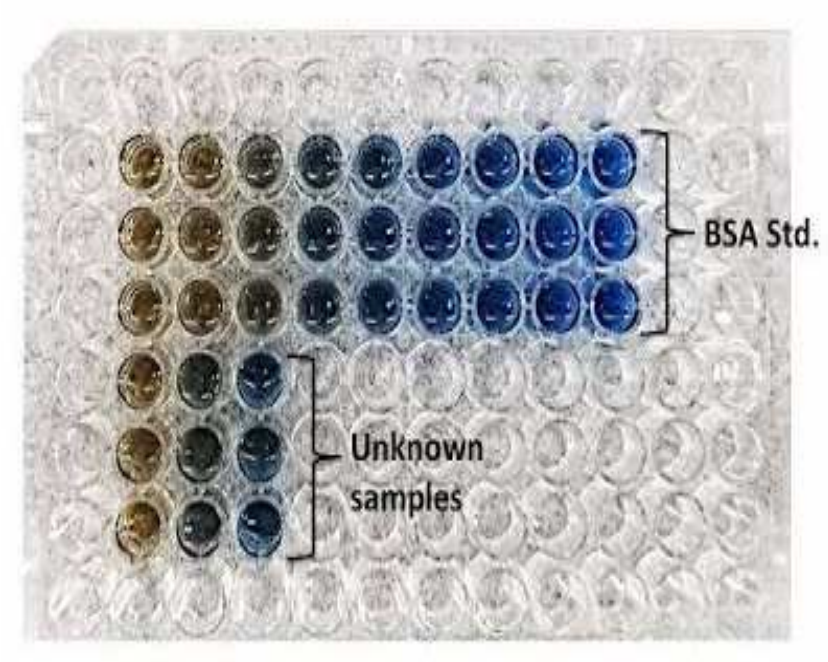


(1) Bradford, Marion M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248_254

Bradford Procedure

- Prepare your BSA (Bovine Serum Albumin) standards (mg/ml) in the same buffer as your protein sample. (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7) Working range = 100-1500 µg/mL
- Mix the Bradford reagent solution immediately before use by gently inverting the bottle several times. Do not shake the bottle to mix the solution.
- Remove the amount of reagent needed and equilibrate it to room temperature before use.
- Pipette 5 µL of each standard or unknown sample into the appropriate microplate wells.
- Add 250 µL of the Bradford reagent to each well, then mix by pipetting up and down a few times.
- Incubate the plate for 10 minutes at RT.
- Measure the absorbance at 595 nm with a plate reader.
- Prepare a standard curve by plotting the average blank-corrected 595 nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.



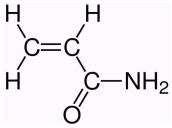
SDS-PAGE Background

Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis is a way to separate proteins by size. The fundamental concept is in an electrophoretic separation, charged molecules are caused to migrate toward the electrode of opposite sign under the influence of an externally applied electric field. The movements of the molecules is retarded by interactions with the surrounding gel matrix, which acts as a molecular sieve. The result is separation by charge and size. To eliminate the charge variable, SDS is added to the sample. SDS will bind tightly to the proteins at about 1.4 mg of SDS/mg of protein. This imparts a negative charge to the protein as well as disrupting tertiary structure.

Disulfide bonds between cystines are not denatured by SDS so a reducing agent such as β-mercaptoethanol (βME) or dithiothreitol (DTT) is added to the samples before electrophoresis. Thus with SDS and βME proteins will be separated by their molecular weight. Low molecular-weight proteins will migrate faster than large ones.

Most electrophoresis is performed in vertical chambers in gel slabs formed between two glass plates. The slab format provides uniformity, so that different samples can be directly compared in the same gel. Gel thicknesses are determined by spacers placed between the glass plates. Wells are formed during polymerization with comb shaped inserts.

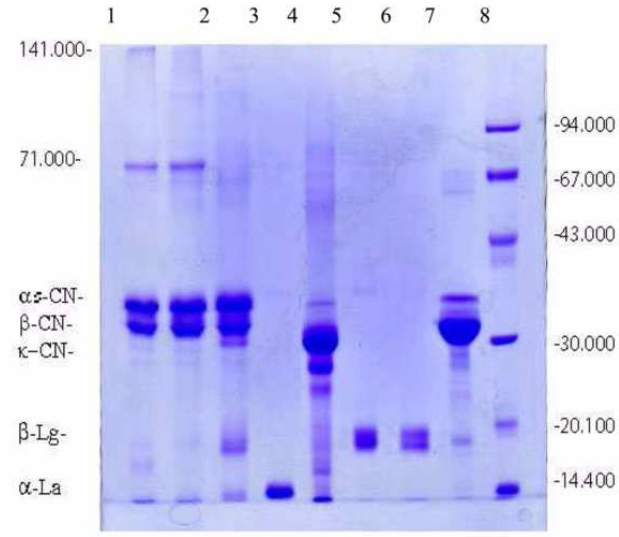
Gels are formed by the copolymerization of acrylamide monomer, and a cross-linking comonomer, *N,N'*-methelenebisacrylamide.



CH_2=CH-CO-NH-CH_2-NH-CO-CH_2

The polymerization of catalyzed by a free radical-generating system composed of ammonium persulfate (the initiator) and an accelerator, tetramethylethylenediamine (TEMED). TEMED causes the formation of free radicals from persulfate and these in turn catalyze polymerization of free radicals from persulfate and these in turn catalyze polymerization.

The sieving properties of a gel are established by the three-dimensional network of fibers and pores which are formed as the gel polymerizes. Within limits, by adjusting the acrylamide concentration the pore sizes are changed. An increased acrylamide concentration results in decreased pore sizes.



In SDS-PAGE the quality of the components is of prime importance to achieve reproducible gels. Chilling the running buffer is suggested to offset the effects of heat during electrophoresis. Even with all precautions, very basic proteins, very acidic proteins, various glycoproteins and lipoproteins, will migrate "anomalously".

The most used method (SDS-PAGE) was developed by Laemmli (2). This is a discontinuous system consisting of two contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different acrylamide concentrations. The sample is first mixed with a loading buffer that makes it more dense than the running buffer. Thus the sample will 'fall' down into the well. As the sample electrophoretically enters the separating gel it slows down but the sample still in the well moves faster. The result is a compression of the sample as it enters the separating gel. Continuing, as the sample leaves the lower acrylamide concentration stacking gel, it encounters the higher acrylamide concentration (thus smaller pore size) resolving gel. Again the sample band is further compressed thus creating narrower bands.

Warning: in the unpolymerized state acrylamide is a neurotoxin. Use precautions. Once polymerized, acrylamide is safe to handle.

(2) U.K. Laemmli, *Nature(London)* 227: 680 (1970)

Pouring SDS-PAGE Gels

- Obtain a large and a small glass plate.
- Make sure the plates are thoroughly cleaned.
  Wash in water with detergent, rinse with DW, ethanol, and finally with acetone to evaporate residual water spots. Make sure plates are dry.
- Place the small plate on top of the larger plate with the attached spacers between (defines the thickness)
- Place the glass plates in the clamp bracket.
- Stand it all vertically on the bench top (to get an even bottom) and clamp everything together.
- Set the glass and clamp bracket into the cradle stand with the bottom pressed into the gasket.
- Prepare the resolving gel in a small beaker.
  Add the TEMED last.
- Transfer the resolving gel to between the glass plates with a Pasteur pipet.
- Carefully cover the gel with water (so you get a smooth top).
- Wait until the gel polymerizes. You should see a sharp line between the gel and the water.
- Pour off the water and prepare the stacking gel and transfer as above.
- Push the comb into the gel sandwich.
- Allow the stacking gel to polymerize.

Mini SDS-PAGE Gel Recipes

Resolving Gel	6.0%	7.5%	10.0%	12.0%	15.0%
DDW	11.7 ml	10.85 ml	9.7 ml	8.7 ml	7.2 ml
40 % acrylamide/BIS	3.0 ml	3.75 ml	5.0 ml	6.0 ml	7.5 ml
1.5 M Tris pH 8.5	5.0 ml				
20 % SDS	100.0 µl				
10 % Ammon. persulfate	200.0 µl				
TEMED	8.0 µl				

Stacking Gel	1 Gel	2 Gels
DDW	2.44 ml	3.66 ml
40 % acrylamide/BIS	0.5 ml	0.75 ml
0.5 M Tris pH 6.8	1.0 ml	1.5 ml
20 % SDS	20.0 µl	30.0 µl
10% Ammon. persulfate	40.0 µl	60.0 µl
TEMED	4.0 µl	6.0 µl

Running Buffer	600 ml	1 Liter
Tris Base	9.0 g	15.0 g
Glycine	43.2 g	72.0 g
SDS	3.0 g	5.0 g

Electrophorese @ 100 - 150 constant volts