OTHER PROTEIN CONCENTRATION ASSAYS

Introduction

While UV-Vis absorption at 280 nm is the most common way of measuring protein concentration (see our protocol on Determining Protein Concentration by UV), this method cannot always be used. UV-Vis absorption is better for purer protein samples and often is not suitable or accurate for solutions containing mixtures of proteins.

Other options that use a UV or fluorescence spectrometer are available and the main ones are presented in this protocol. These protein assay methods can be split into three categories: protein-copper interaction analysis, protein-dye interaction analysis and fluorescence-based analysis.

See also our protocol on determining protein concentration by amino acid analysis.

Theory

Protein-Copper based protein assays

Biuret Reaction

The Biuret reaction underpins all copper-based protein assays, such as the Bicinchoninic Acid (BCA) and Lowry protein assays. Where peptide chains larger than tripeptides or proteins are incubated with an alkaline copper (Cu^{2+}) solution, a complex made up of the copper ion and 4-6 peptide bonds is formed. The Cu^{2+} ion is reduced to Cu^{1+} and the complex formed will be a light blue to violet colour. The intensity of this colour will be proportional to the number of peptide bonds and absorbs light at 540 nm.

Bicinchoninic Acid (BCA) Protein Assay

The first step of the BCA protein assay is the Biuret reaction. BCA (bicinchoninic acid) is then added and reacts with the Cu^{1+} ion formed as a result of the Biuret reaction. Two BCA molecules chelate to each Cu^{1+} ion. This complex has a purple colour and shows a strong linear absorbance at 562 nm. Cysteine, tyrosine, and tryptophan have been shown to enhance Cu^{2+} reduction to Cu^{1+} and so increase the amount of BCA-Cu^{1+} complexes formed. However, the main bulk of BCA-Cu^{1+} complexes formed are a result of the Cu^{2+} Biuret reduction to Cu^{1+} from the peptide bonds.

Lowry Protein Assay

The mechanism of colour formation in the Lowry protein assay is believed to be similar to that of the BCA assay, but it is not as well understood. Once the protein is incubated with an alkaline $CuSO_4$ in the presence of tartare, a treadentate copper complex forms. This is similar to the Biuret reaction. The second reagent, Folin-phenol, is added and reduced, forming an intense blue complex. This reduction

is believed to occur when the tetradentate copper complex transfers electrons to the Folin-phenol reagent, though the mechanism is currently unknown. The reduced Folin-phenol reagent shows a strong absorbance at 750 nm. Five amino acids – tyrosine, tryptophan, cysteine, histidine, and asparagine – also act as reducing agents, therefore enhancing the reduction of the Folin-phenol reagent.

Protein-dye based protein assays

Coomassie Dye (Bradford) Protein Assay

Coomassie dye, while in an acidic environment, will bind to proteins. Coomassie dye is generally a red-brown colour which has an absorbance maximum at 465 nm. When bound to the protein, the Coomassie dye takes on a blue colour with an absorbance maximum at 610 nm. The difference between the two colours of the dye is greatest at 595 nm and so this is the optimal wavelength to measure the blue colour from the protein-Coomassie dye complex. This blue colour is believed to be a result of interactions between the dye and basic amino acids within the protein, particularly arginine, lysine, and histidine. Van der Waals and hydrophobic interactions are also involved in binding the protein being assayed and the Coomassie dye.

Fluorescence based Protein Assays

There are slight differences in the mechanism of action between different fluorescent protein assays, but they work via the same general theory. When the dye is added to the protein solution, the dye and the protein bind, resulting in a fluorescent signal being emitted. Any dye that is not bound to the protein will not be fluorescent and so the strength of the fluorescent signal is proportional to the amount of protein in the solution. This fluorescent signal can be read by a fluorometer, and its intensity measured.

Methods

Standard curve

The assays can be used to determine the protein concentration of a heterogeneous or a pure protein sample. In both cases a standard curve should be generated in order to reduce any errors from the amino acid composition and/or buffer components.

- Solutions containing known protein concentrations (standards) should be made up and treated in the same way as the protein being assayed. i.e they should, where possible be in the same buffer.
- Standard samples should be made up to cover the linear range of the particular assay being used and should be at evenly spaced concentrations. For example: 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mg/mL.
- Ideally, standard samples should be run in duplicate or triplicate
- The best protein to use as a standard is a pure version of the protein in question but often this is not possible
- Two standards commonly used are bovine serum albumin (BSA) or bovine gamma globulin (BGG). Stock solutions of known concentration should be purchased.
- BGG should be used as a standard when determining the concentration of antibodies
- For protein assays where protein to protein variation is really high, BSA can give misleading results if the protein in question has a sequence very different to the BSA sequence – in those cases, proteins with a similar sequence are better standards

BCA protein assay

The BCA reaction is temperature dependent and at higher temperatures, more colour formation is seen due to the higher reactivity of the main components of the BCA reaction – tryptophan, tyrosine and the peptide bonds. Considerations for the buffer components are given in table 1 below.

While different commercial sources will have their own recommended procedures, a general BCA method would be:

1. Make up the protein samples and standard samples at the same time (these should be in the linear range of the particular BCA kit you are using)
    a. For a cuvette analysis 100 μL of protein sample and 2 mL of BCA working reagent should be used
    b. For a 96 well plate analysis 25 μL of protein and 200 μL of BCA working reagent should be used
2. Incubate the sample and standards at either 37 °C or 60 °C for 30 minutes
3. Allow the sample to equilibrate to room temperature before measuring the absorbance

4. The absorbance should be measured at a wavelength of 562 nm
5. The BCA assay should show a linear response over a wide concentration range

Note: If incubation is taking place at 37 °C a longer incubation period should improve protein to protein variability

Lowry protein assay

While there are many modified versions of the Lowry assay available commercially, a general method for the Lowry assay would be:

Considerations for the buffer components are given in table 1 below.

1. Make up the protein samples and standard samples at the same time (these should be in the linear range of the particular Lowry kit you are using, typically 5 - 100 μg/mL)
    a. For a cuvette analysis 1 mL of protein sample and 1 mL of the alkaline copper reagent should be added, mixed and allowed to stand for 10 minutes
    b. For a 96 well plate analysis 100 μL of protein and 100 μL of the alkaline copper reagent should be added, mixed and allowed to stand for 10 minutes
2. 0.5 mL of Folin-phenol reagent should be added (or 50 μL for plate assay) and the mixture should be vortexed and incubated for 30 minutes
3. The mixture should be vortexed again and the absorbance should be measured at a wavelength of 750 nm
4. The Lowry assay should show a linear response over a limited range of standards, but different models can also be used

Bradford protein assay

Bradford assay kits can be purchased. Alternatively the dye can be made up as follows: 100 mg Coomassie Brilliant Blue G-250 should be dissolved in 50 mL of 95% ethanol and 100 mL of 85% phosphoric acid should be added while stirring constantly. The dye should then be diluted to 1 L in deionised water.

Considerations for the buffer components are given in table 1 below.

1. Make up the protein samples and standard samples at the same time (these should be in the linear range of the particular Bradford kit you are using, typically 100 – 1500 μg/mL
2. The protein and standard samples should be added to disposable cuvettes (the dye will stick to various surfaces)
3. The Bradford reagent should be at room temperature
    a. For a cuvette analysis 50 μL of protein sample and 2 mL of the dye solution should be added, mixed and allowed to stand for 10 minutes

b. For a 96 well plate analysis 25 μL of protein and 200 μL of the dye solution should be used, mixed and allowed to stand for 10 minutes

4. The absorbance should be measured at wavelengths of 450 and 595 nm
5. Either the 595 nm data should be plotted, or the 595 nm/450 nm ratio should be plotted on a graph
6. The 595 nm/450 nm ratio will show better precision at a lower response
7. The Bradford asay should show a polynomial response

Fluorescence based assay

- There are many different commercial fluorescence dyes and fluorometers available and all have different recommended methods
- Generally, a fluorometer will need to be calibrated before use
- The protein and fluresence dye will be mixed together and pipetted onto a plate reader
- The plate reader will be placed into the fluorometer and the intensity of the fluorescence will be measured
- Using a standard curve, the concentration of the desired protein can be calculated
- Typical working ranges for fluorescent assays are 10 ng/mL to 150 μg/mL and 50 μg/mL to 5 mg/mL
- Fluorescent assays are very sensitive so less protein is required for quantitation and there is low protein to protein variation

Table 1: Buffer components that can affect BCA, Lowry and Bradford assays

Reagent	Reagent conc'n above which will disrupt……		
	BCA protein assay	Lowry protein assay	Bradford protein assay
Acids/Bases			
HCl	100 mM	N/A	100 mM
NaOH	100 mM	N/A	100 mM
Buffers/Salts			
Glycine	1 mM	1 mM	100 mM
Imidazole	50 mM	25 mM	200 mM
HEPES	100 mM	1 mM	100 mM
Ammonium sulphate	1.5 M	>28 mM	1 M
NaCl	1 M	1 M	5 M
Sodium azide	0.2%	0.5%	0.5%
Detergents			
CHAPS	5%	0.06%	5%
Triton X-100	5%	0.03%	0.13%
Tween20	5%	0.06%	0.06%
SDS	5%	1%	0.13%
Solvents			
Glycerol	10%	10%	10%
Urea	3 M	3 M	3 M
PMSF	1 mM	1 mM	1 mM
DMSO	10%	10%	10%
Ethanol	10%	10%	10%
Sucrose	40%	7.5%	10%
Guanidine-HCl	4 M	100 mM	3.5 M
Chelating agents			
EDTA	10 mM	1 mM	100 mM
Reducing agents			
DTT	1 mM	0.05 mM	5-1000 mM
β-Mercaptoethanol	0.01%	1 mM	1 M

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Pros and Cons of the various protein assays

Protein Assay	Advantages	Disadvantages
BCA	♦ Compatible with protein samples containing up to 5% detergents ♦ A linear response is seen ♦ Less protein to protein variation seen than with the Bradford assay	♦ Identical incubation times for protein sample and standards are required as the BCA assay does not have an end point ♦ Chelating agents can interfere with the amount of copper available for the Biuret reaction ♦ Copper reductants will interfere with the BCA assay ♦ Incubation temperatures are important for the BCA assay – either 37 °C or 60 °C
Lowry	♦ A linear response is seen ♦ Less protein to protein variation seen than with the Bradford assay	♦ Incompatible with detergents and potassium ions ♦ Chelating agents can interfere with the amount of copper available for the Biuret reaction ♦ Reducing agents and free thiols will reduce the Folin-phenol reagent ♦ The Folin-phenol reagent has to be added after precisely 10 minutes and the mixture has to be vigorously stirred to prevent the alkaline pH of the Lowry reagent from deactivating the Folin-phenol reagent
Bradford	♦ Performed at room temperature ♦ Very fast compared to BCA and Lowry assays ♦ No special equipment is required ♦ Compatible with most salts, solvents, buffers, thiols, reducing agents and chelating agents	♦ Incompatible with detergents ♦ High protein to protein variation ♦ Coomassie dye is very acidic so proteins must be compatible with low pH environments
Fluorescent	♦ High sensitivity, so little protein has to be used ♦ Timing is not critical unlike the BCA, Lowry and Bradford assays ♦ Low protein to protein variation	♦ Samples may need to be diluted until a reasonable reading is seen ♦ Specialised equipment, like a fluorometer, is required ♦ Different commercial fluorescent dyes have different compatibilities with substances found in protein buffers – compatibilities have to be checked on an individual basis

References

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