Technical Note: Colorimetric Protein Assays

Assays for Determining Protein Concentration

Contents

Introduction

Multiple colorimetric protein assays exist for determination of protein concentration, differing in sensitivity, specificity, and speed. Accuracy is critical for downstream processes, as errors in protein concentration can greatly influence any inferences drawn from these processes. No one assay yields totally accurate results, and each has its advantages and disadvantages. The most common protein assays, BCA and Biuret (Smith *et al*., 1985), Bradford (Bradford, 1976), Lowry (Lowry *et al*., 1951), and variants thereof, are frequently purchased as commercial kits and as a result their limitations and suitability are often overlooked.

Assay Choice

For many end-users their choice of assay depends on its sensitivity, protocol simplicity, and speed. No colorimetric assay is as simple and quick as direct UV measurements at 280 nm for the determination of protein concentration. However measurements at 280 nm rely on the protein containing aromatic amino acids such as tyrosine (Y), phenylalanine (F), and, or tryptophan (W). Not all proteins contain these amino acids, and the relative proportions of theses amino acids differ between proteins. Furthermore, if nucleic acids are present in the sample, they would also absorb light at 280 nm, further compromising accuracy. Therefore, the sensitivity achieved without prior knowledge of the protein of interest's absorbance maxima (λ_{max}) and attenuation coefficient (ε), or the samples purity, demonstrates the colorimetric assays fundamental advantage of direct UV measurements.

The differences between each assay is relatively apparent (Table 1). Therefore, careful considerations should be made to ensure appropriate results are achieved.

Buffer Composition

The sample buffer composition is an important consideration when selecting a protein assay (Table 2). Chromophore development in the Biuret assay, and subsequently the BCA and Lowry assays, is subject to interference from metal chelators, such as ethylenediamine, and ethyleneglycoldiamine, tetraacetic acid (EDTA and EGTA). The BCA assay, when compared to the Lowry assay, is compatible with a wide range of detergents, including sodium dodecylsulphate

Table 1: Colorimetric Protein Assays

	Biuret Assay	BCA Assay	Lowry Assay
Active Reagent	Biuret reagent	Bicinchoninic acid	Folin–Ciocalteu reagent
Mechanism	$Cu^{2+} \rightarrow Cu^{1+}$ reduction	Detection of Cu^{1+} from Biuret assay	Detection of Cu^{1+} from Biuret assay
Protein target	Peptide chain		
Wavelength	546 nm	562 nm	750 nm
Sensitivity	5 – 160 mg/mL	0.0005 – 2 mg/mL	0.005 – 2 mg/mL
Advantages	Detects peptides as small as 3 amino acids, sequence dependant		
Disadvantages	Interference from reducing agents, metal chelators, thiol containing and aromatic amino acids		
Bradford Assay			
Active Reagent	Coomassie Brilliant Blue G-250		
Mechanism	Ionic interactions between dye sulfonic acid groups and positive protein amine groups		
Protein target	Arginine and to a lesser extent histidine and lysine		
Wavelength	595 nm		
Sensitivity	0.001 – 2 mg/mL		
Advantages	Broad buffer compatibility, quick and simple		
Disadvantages	Interference from basic and aromatic amino acids, non-linear response, cannot detect proteins <3 kDa		

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Table 2: Some Common Buffer Component Tolerances

	Component	Concentration Limit		
		BCA	Lowry	Bradford
Acids and Bases	HCl	0.1 M	-	0.1 M
	NaOH	0.1 M	-	0.1 M
	PCA	0.1 M	0.125 M	-
	TCA	60 mM	80 mM	-
Buffers	Acetate	0.2 M	-	0.6 M
	Ammonium sulphate	1.5 M	28 mM	1 M
	Borate	10 mM	-	-
	Citrate	1 mM	2.5 mM	50 mM
	Glycine	1 M	2.5 mM	0.1 M
	HEPES	0.1 mM	2.5 µM	0.1 M
	Phosphate	0.25 M	0.25 M	2 M
	TRIS	0.1 M	0.25 M	2 M
Detergents	Brij 35	1 %	-	-
	CHAPS	1 %	-	-
	Deoxycholate	-	0.0625 %	0.25 %
	Lubrol PX	1 %	-	-
	Octylglucoside	1 %	-	-
	SDS	1 %	1.25 %	0.1 %
	Triton X-100	1 %	0.25 %	0.1 %
	Tween 20	1 %	0.1 %	-
Reductants	DTT	1 mM	50 µM	1 M
	βME	0.1 M	1.8 µM	1 M
Miscellaneous	Nucleic Acids	2 µg/µL	0.2 µg/µL	10 µg/µL
	DMSO	5 %	6.2 %	-
	EDTA	10 mM	0.125 mM	0.1 M
	Glycerol	10 %	25 %	100 %
	KCl	10 mM	30 mM	1 M
	NaCl	1 M	1.75 M	5 M
	Sucrose	1.2 M	50 mM	1 M
	Urea	3 M	0.2 M	6 M

Source: Increased uniformity in the response of the Coomassie blue G protein assay to different proteins (1990). Abbreviations: HCl, hydrochloric acid; NaOH, sodium hydroxide; PCA, perchloric acid; TCA, trichloroaceitic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TRIS, 2-Amino-2-(hydroxymethyl) propane-1,3-diol; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulphonate; SDS, sodium dodecyl sulphate; DTT, (2S,3S)-1,4-Bis(sulfanyl)butane-2,3-diol; βME, 2-Sulfanylethan-1-ol; DMSO, Dimethyl sulphoxide; EDTA, 2,2',2",2"'-(Ethane-1,2-diyldinitrilo)tetraacetic acid; KCl, potassium chloride; NaCl, sodium chloride.

(SDS), but is more susceptible interference from reducing agents such as dithiothreitol (DTT). The Bradford assay is not compatible with high detergent concentrations but tolerates reducing agents, such as DTT or 2-Mercaptoethanol (2-ME). For the best results, incompatible buffers should be dialysed against, or the protein of interest precipitated and re-suspended in, an appropriate buffer.

Sample Composition

Obvious differences in concentration estimates can be obtained, for the same protein of interest, using different assays. A protein rich in arginine residues will give higher concentration values using a Bradford assay, compared to when using the Lowry or BCA assay. Alternatively, a protein rich in cysteine residues will result in higher concentration values using the BCA assay, compared to when using the Lowry or Bradford assay.

Where possible, creating standard curves using the protein to be assayed, or at least one with similar proportions of interfering amino acids, helps to minimise this effect. When the sample consists of complex protein mixtures, it is generally considered that the BCA and Lowry assays perform better.

Standard Selection

The protein used in the standards is important. For the best results, calibration should be carried out with the same protein to be assayed. Where this is not feasible alternative protein standards can be used. Bovine serum albumin (BSA) tends to be the standard of choice, and is commonly supplied with commercial kits. However, deviations can be observed using various standards with the Bradford assay, with BSA having a significantly higher than expected response (Stoscheck, 1990). Therefore, immunoglobulin G (IgG) or lysozyme are preferred standards for the assay. Alternative standards should always be considered based on the amino acid composition of the protein of interest. The Bradford assay will overestimate the concentration of proteins rich in arginine residues, whereas Biuret, BCA and Lowry assays will overestimate the concentration of proteins rich in thiol containing cysteine residues. Generally however, it is considered that the BCA and Lowry assays perform better with complex protein mixtures.

Assays

Each assay protocol is set around mixing the active reagent with a series of standard protein samples of known concentration and measuring the colorimetric response. A standard curve is then created by plotting the absorbance against the concertation of the standards. This standard curve acts as a calibration curve form subsequent measurements of protein sample of unknown concertation.

Additional steps specific to the protocol followed may be included, such as incubation of the mixture in the case of BCA, Biuret, and Lowry, and variation exists between different protocols of the same assay.

Biuret, BCA, and Lowry Assays

Also known as the Piotrowski test, the Biuret reagent is made of sodium hydroxide (NaOH), hydrated copper (II) sulphate, and potassium sodium tartrate which chelates and stabilise the cupric ions. Under the alkaline conditions, the reaction between the cupric ions and the nitrogen of the peptide bonds, leads to the displacement the peptide hydrogen and to the reduction of Cu^{2+} to Cu^{1+}. A tri- or tetradentate chelation with nitrogen produces the characteristic mauve colour, which is detectable at or near 550 nm (Figure 1). The mechanism is sensitive enough to detect dipeptides, a peptide composed of two amino-acid residues joined by a single peptide bond (Datta *et al.,* 1959).

The BCA and Lowry assays offer two modifications to the biuret assay and are more common in modern peptide and protein analysis. In these assays, the Cu^{1+} formed during the biuret reaction reacts further with other reagents, leading to a deeper colour.

In the BCA assay, Cu^{1+} forms a deep purple complex with BCA which absorbs around 562 nm (Smith *et al*., 1985). The water-soluble BCA-copper complex absorbs more strongly than the peptide-copper complex, increasing the sensitivity of the biuret assay from 5 mg/mL to 0.0005 mg/mL. Additionally, benefits are the BCA assays compatibility with a broader range of buffer components.

In the Lowry assay Cu^{1+} is oxidized back to Cu^{2+} by molybdenum(VI) (Mo^{6+}) in Folin-Ciocalteu's reagent, which forms molybdenum blue (Mo^{4+}). The additional chemistry results in an increase of protein detection sensitivity to as low as 0.005 mg/mL (Lowry *et al*., 1951). Additional processing of molybdenum blue with organic dyes such as malachite green and Auramine O, can be used to further amplify the signal by up to fifty times (Sargent, 1987).

Bradford Assay

The Bradford assay is based on a λ_{max} shift of the Coomassie Brilliant Blue G-250 (CBBG) dye, from the anionic blue (595 nm) form, to the neutral green and cationic red (465 nm) forms (Figure 2). Under acidic conditions, the red form acts as a reducing agent and is converted to its blue form, stabilised by binding to the basic amino acid (histidine, lysine, and most notably arginine) carboxyl groups via Van der Waals force and amino groups through electrostatic interactions (Ninfa, Ballou and Benore, 2010).

Unlike other protein assays, the Bradford protein assay is less susceptible to interference by various chemical compounds (Barbosa, Slater and Marcos, 2009; Bradford, 1976; Ninfa, Ballou and Benore, 2010). A significant exception is detergents, for example, concentrations of SDS below the critical micelle concentration (CMC) of 0.00333 % to 0.0667 %, binds strongly to the protein, inhibiting dye binding. This can result in the protein concentration being underestimated. While SDS concentrations above the CMC

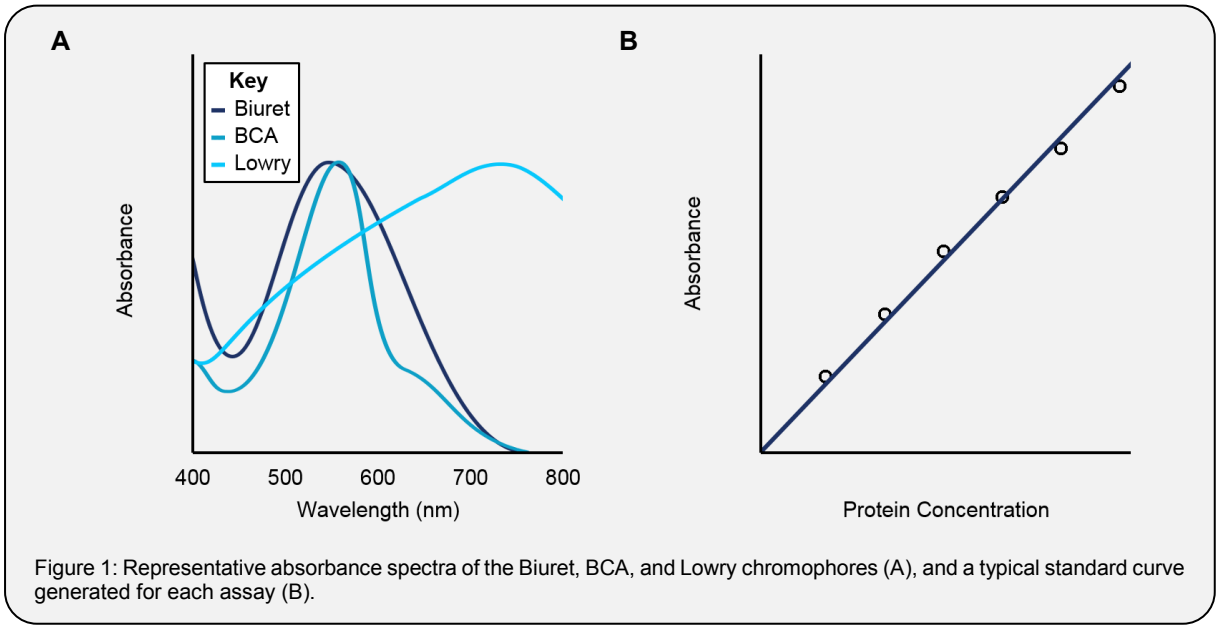

Figure 1: Representative absorbance spectra of the Biuret, BCA, and Lowry chromophores (A), and a typical standard curve generated for each assay (B).

associates strongly with the green form of the CBBG dye. This causes a shift towards the blue form and can result in overestimation of the protein concentration. The inhibition by detergents has been shown to be alleviated by the addition of cyclodextrins to the assay mixture (Rabilloud, 2016; Rabilloud, 2018).

The Bradford assay is one of the fastest assays performed on proteins (Okutucu et al., 2007), with a total time to set up and complete being under 30 minutes. Furthermore, Bradford reagent can remain stable for two weeks and the entire experiment is done at room temperature. Its sensitivity is also comparable, 0.001 mg/mL, to the BCA and Lowry assays.

Due to the relative content of arginine in proteins, the Bradford assay typically struggles to detect proteins smaller than 3 kDa. An additional disadvantage is that the concentration response of the standard curve is not linear. Largely due to the equilibrium between the two forms of the dye being disturbed in the presence of protein. The modified Bradford assay is made linear by plotting the A595/A465 ratio over the protein standard concentration, and is approximately 10 times more sensitive than the conventional assay (Zor and Selinger, 1996).

Troubleshooting

Unexpected concentration estimates can be seen when the protein concentration falls outside of the assays detection limit. Specifically, if the protein is small, less than 3 to 5 kDa, the Bradford assay may not be able to detect it.

If the estimated concentration falls outside of the range of the standard curve, the amount of protein used during the assay should be adjusted accordingly. This can be avoided by preparing a range samples containing various amounts (protein volume per assay volume) of the protein of interest. The concentration can then be divided by the protein volume and then averaged across the range (Table 3). Other factors include incubation time and temperature, and wavelength. If the assay is not performed under the same conditions used to create the standard curve, the concentration estimates can be affected. It is good practice to run a standard alongside the protein of interest to see if it correlates with the standard curve.

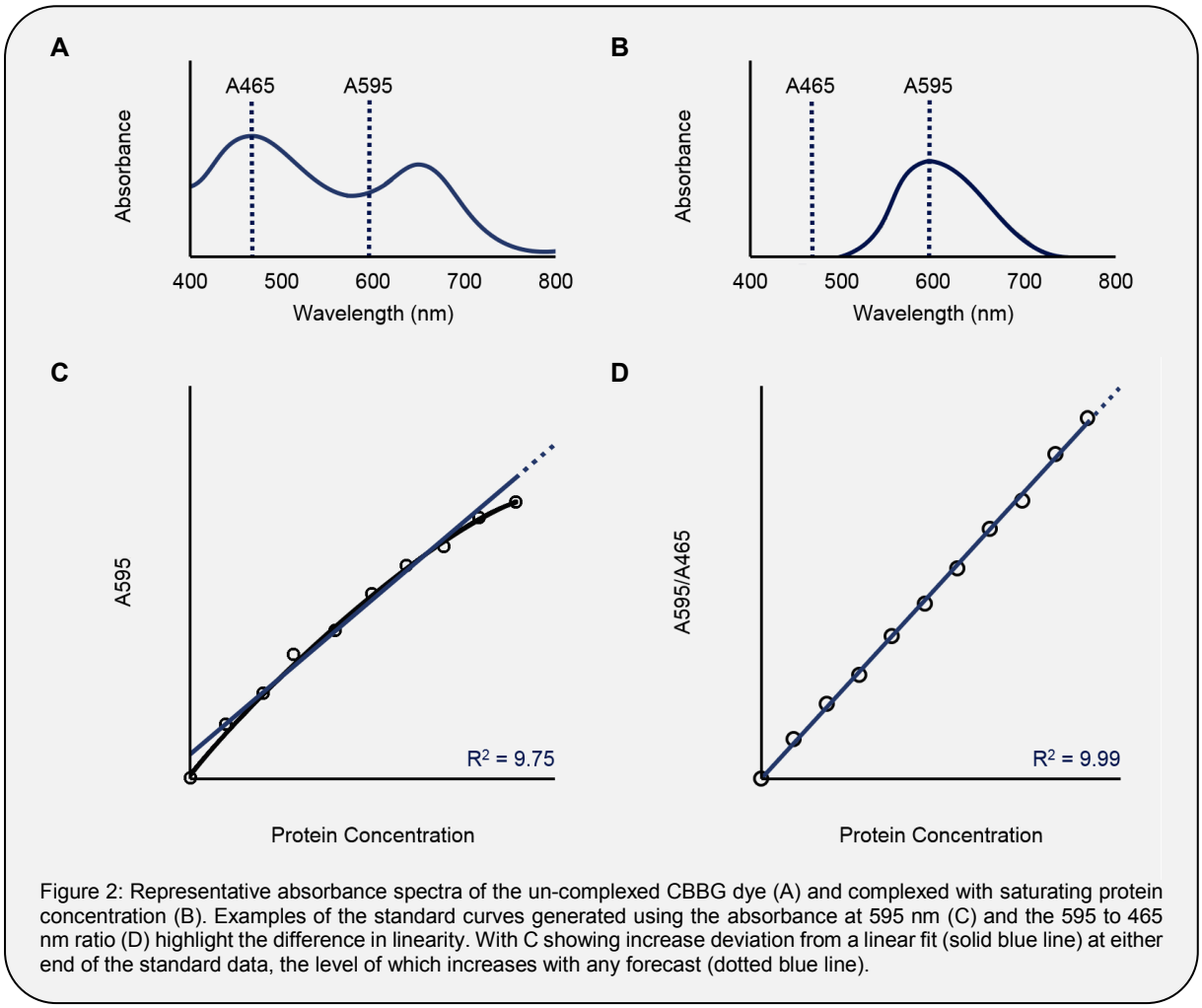

Figure 2: Representative absorbance spectra of the un-complexed CBBG dye (A) and complexed with saturating protein concentration (B). Examples of the standard curves generated using the absorbance at 595 nm (C) and the 595 to 465 nm ratio (D) highlight the difference in linearity. With C showing increase deviation from a linear fit (solid blue line) at either end of the standard data, the level of which increases with any forecast (dotted blue line).

Table 3: Sample Ranges of Varying Protein Content

Protein (µL)	Absorbance (AU)	Concentration (mg/mL)	$\frac{Concentration}{Protein\ (\mu L)}$
0	0.00	0.00	-
1	0.09	1.15	1.15 mg/mL
5	0.43	5.49	1.10 mg/mL
10	0.87	10.99	1.10 mg/mL
20	1.72	21.98	1.10 mg/mL
		Average	**1.11 mg/mL**

If the absorbance values of the standard curve are lower than expected, most commonly the dye reagents need replacing as a result of aging or improper storage. Alternatively the standard dilutions may not have been prepared correctly.

Finally, check the protein buffer for the presence of interfering substances as described in this document. Ultimately, where possible, the protocol being worked from should be followed to as closely as possible to achieve comparable results.

FAQ's

Q1 Can the same standard curve be used for different proteins?

A1 Optimally, calibration should be carried out with the same protein to be assayed. However, as Biuret, BCA, and Lowry assays have little dependency on the amino acid sequence, and produce a linear standard curve, a correction factor can be determined.

$$k = \frac{P_1}{P_2}$$

Where k is the correction factor, P_2 is the absorbance for sample of known concentration of the protein of interest, and P_1 is the absorbance at the same concentration from the existing standard curve. This can then be applied to the standard curve line equation. For example, if the protein of interest gives an absorbance value of 0.5 AU for a 2 mg/mL sample. The absorbance for a 2 mg/mL sample is calculated from the existing standard curve line equation, following linear regression.

$$y = mx + c$$

Where y is the absorbance (AU), m is the gradient of the line, x is the concentration (mg/mL), and c is the y-intercept of the line.

e.g.

$$y = 0.35 \times 2 + 0 = 0.7$$

The correction factor is then determined.

$$k = \frac{0.7\ AU}{0.5\ AU} = 1.4$$

Which can be applied to the rearranged standard curve line equation for determining the concentration of the protein of interest.

$$x = \left(\frac{y - c}{m}\right) \times k$$

e.g.

$$x = \left(\frac{0.5 - 0}{0.35}\right) \times 1.4 = 2$$

Q2 Can the assays be performed using micro volume platform instruments and short pathlength cuvettes?

A2 Yes, however the usual limitations of short pathlengths giving less sensitive and less reproducible measurements at lower sample concentrations, especially for larger molecules, exist. Therefore, their suitability should be carefully considered.

Preferably the sample measurements should to be taken at the same pathlength that the standard curve was created with. However a pathlength correction factor (k) can be determined by dividing the original pathlength (l_1) by the new pathlength (l_2).

$$k = \frac{l_1}{l_2}$$

This can then be applied to the rearranged line equation from the standard curve to determine the concentration.

$$x = \left(\frac{y - c}{m}\right) \times k$$

Where x is the concentration (mg/mL), y is the absorbance (AU), c is the y-intercept of the line, and m is the gradient of the line.

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