CHAPTER EIGHT

Quantitation of Protein

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Contents

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Abstract

The measurement of protein concentration in an aqueous sample is an important assay in biochemistry research and development labs for applications ranging from enzymatic studies to providing data for biopharmaceutical lot release. Spectrophotometric protein quantitation assays are methods that use UV and visible spectroscopy to rapidly determine the concentration of protein, relative to a standard, or using an assigned extinction coefficient. Methods are described to provide information on how to analyze protein concentration using UV protein spectroscopy measurements, traditional dye-based absorbance measurements; BCA, Lowry, and Bradford assays and the fluorescent dye-based assays; amine derivatization and detergent partition assays. The observation that no single assay dominates the market is due to specific limitations of certain methods that investigators need to consider before selecting the most appropriate assay for their sample. Many of the dye-based assays have unique chemical mechanisms that are prone to interference from chemicals prevalent in many biological buffer preparations. A discussion of which assays are prone to interference and the selection of alternative methods is included.

1. Introduction

The quantity of protein is an important metric to measure during protein purification, for calculating yields or the mass balance, or determining the specific activity/potency of the target protein. Various platforms and methods are available to quantitate proteins and will be described elsewhere in this volume; however, for this chapter, we will concentrate on spectrophotometric assays of protein in solution that do not require either enzymatic/chemical digestion or separation of the mixture prior to analysis.

The spectrophotometric assays described are UV absorbance methods and dye-binding assays using colorimetric and fluorescent-based detection. In comparison to other methods, these assays can be run at a high throughput, using inexpensive reagents with equipment found in the majority of biochemical laboratories. These spectrophotometric assays require an appropriate protein standard or constituent amino acid sequence information to make a good estimate of concentration. The choice of method used to determine the concentration of a protein or peptide in solution is dependent on many factors that will be discussed. The majority of methods

require a soluble analyte such as peptides, proteins, posttranslationally modified protein (e.g., glycosylated), or chemically modified proteins (e.g., PEGylated). For common protein purification procedures, the flow chart in Fig. 8.1 describes the process of selecting the most appropriate assay, based on key criteria.

Where other protein purification techniques are available or complex buffer systems are present in the sample, refer to Table 8.1, or other reviews (Olson and Markwell, 2007) for assay selection. Other criteria that need to be considered when selecting an assay include:

- *Sample volume*: The amount of material available to analyze, typically fluorescent-based assays display the best sensitivity and dynamic range (see Fig. 8.2). Microplate assays (by using lower assay volumes, thereby less protein sample) show improved sensitivity, typically up to 10-fold when compared with cuvette-based assays.
- *Sample recovery*: If the sample is limited, a nondestructive method, for example, UV spectroscopy may be more appropriate.
- *Throughput*: If multiple samples are to be analyzed, a microplate-compatible rapid one step assay should be considered.
- *Robustness*: The absorbance-based dye-binding assays appear to display enhanced repeatability and robustness when compared to fluorescent assays.
- *Chemical modification*: Covalent modification, for example glycosylation (de Moreno *et al.*, 1986; Fountoulakis *et al.*, 1992) or PEGylation (Noble *et al.*, 2007), can interfere with specific assays.
- *Protein aggregation*: The solubility of a protein in solution, often a problem for membrane proteins, or proteins prone to aggregation can alter the expected response for many assays.

Other protein quantitation methods are becoming more commonly employed in biochemistry laboratories due to automation, regulatory, and sensitivity requirements. Alternative methods not detailed in this chapter include isotope dilution mass spectrometry (ID IC MS/MS) (Burkitt *et al.*, 2008), Kjeldahl nitrogen method, amino acid analysis (Ozols, 1990), gravimetric determination (Blakeley and Zerner, 1975), immunological, and quantitative gel electrophoresis with fluorescent staining.

2. General Instructions for Reagent Preparation

For the methods detailed, reagents should be used at the highest purity available and dyes should be obtained at spectroscopy grade where available. Ideally deionized, filtered water should be used at a minimum quality of

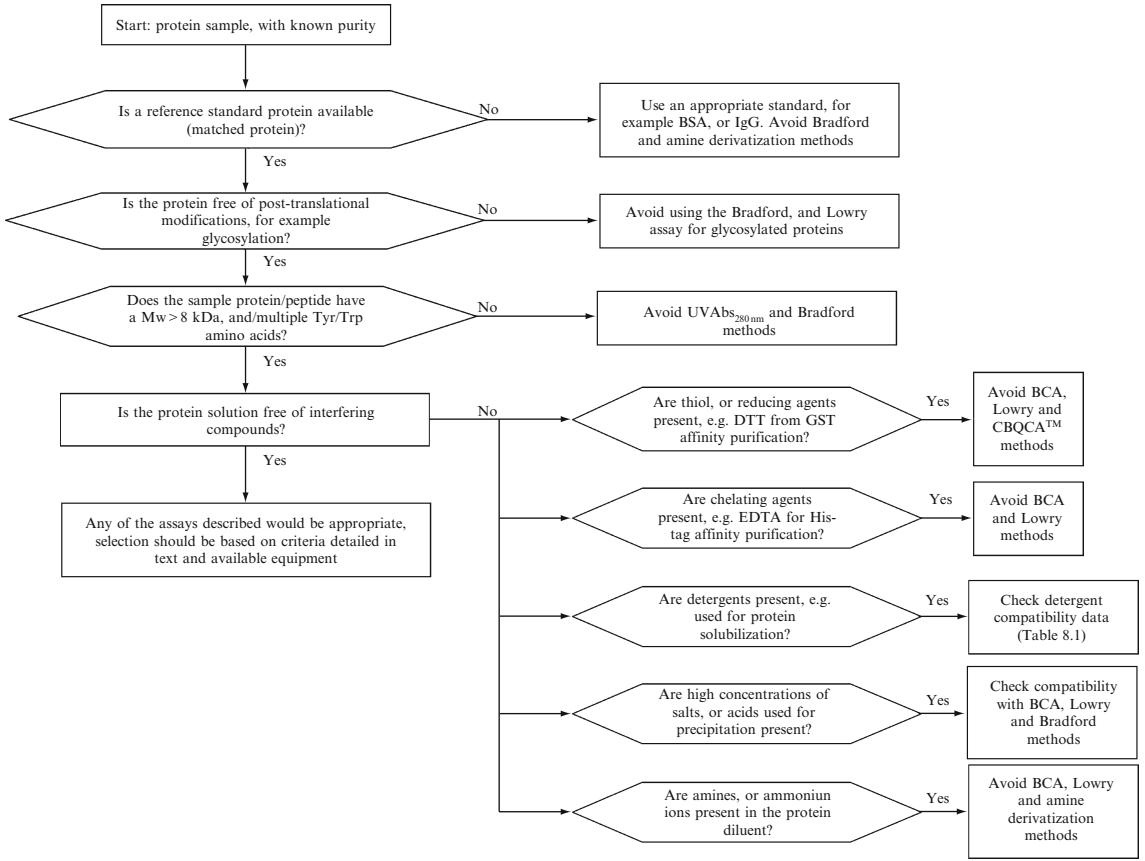

Figure 8.1 Flow chart for the selection of assays for quantitation or proteins in common protein purification procedures. The chart assumes that the sample for analysis is relatively pure, that is the analyte for quantitation is the major component, for example fractions from affinity chromatography, or extraction from inclusion bodies. The ''reference standard protein'' refers to a standard that is the same protein that is being quantitated in the same, or similar matrix that is ''matched.''

Table 8.1 Substance compatibility table

Substance	Compatible concentration[a]					
	BCA[b]	Lowry[c]	Bradford[d]	Amine derivatization[e]	Fluorescent detergent[f]	UV $Abs_{280\ nm}$[g]
Acids/bases						
HCl	0.1 *M*	na	0.1 *M*	na	10 m*M*	>1 *M*
NaOH	0.1 *M*	na	0.1 *M*	na	10 m*M*	>1 *M*
Perchloric acid	<1%	>1.25%	na	na	na	10%
Trichloroacetic acid	<1%	>1.25%	na	na	na	10%
Buffers/salts						
Ammonium sulfate	1.5 *M*	>28 m*M*	1.0 *M*	10 m*M*	10–50 m*M*	>50%
Borate	10 m*M*	Undiluted	Undiluted	Undiluted	Undiluted	Undiluted
Glycine	1 m*M*	1 m*M*	100 m*M*	–	na	1 *M*
HEPES	100 m*M*	1 m*M*	100 m*M*	na	10–50 m*M*	na
Imidazole	50 m*M*	25 m*M*	200 m*M*	na	na	na
Potassium chloride	<10 m*M*	30 m*M*	1.0 *M*	na	20–200 m*M*	100 m*M*
PBS	Undiluted	Undiluted	Undiluted	Undiluted	Undiluted	Undiluted
Sodium acetate	200 m*M*	200 m*M*	180 m*M*	na	na	na
Sodium azide	0.2%	0.5%	0.5%	0.1%	10 m*M*	na
Sodium chloride	1.0 *M*	1.0 *M*	5.0 *M*	na	20–200 m*M*	>1 *M*
Triethanolamine	25 m*M*	100 m*M*	na	na	na	na
Tris	250 m*M*	10 m*M*	2.0 *M*	10 m*M*	na	0.5 *M*
Detergents						
Brij 35	5%	0.031%	0.125%	na	na	1%
CHAPS	5%	0.0625%	5%	na	na	10%

(*continued*)

Table 8.1 (*continued*)

Substance	Compatible concentration[a]					
	BCA[b]	Lowry[c]	Bradford[d]	Amine derivatization[e]	Fluorescent detergent[f]	UV $Abs_{280\ nm}$[g]
Deoxycholic acid	5%	625 μg/ml	0.05%	na	na	0.3%
Nonidet P-40	5%	0.016%	0.5%	na	na	na
SDS	5%	1%	0.125%	–	0.01–0.1%	0.1%
Triton X-100	5%	0.031%	0.125%	na	0.001%	0.02%
Tween-20	5%	0.062%	0.062%	0.1%	0.001%	0.3%
Reducing agents						
Cysteine	na	1 m*M*	10 m*M*	na	na	na
DTT	1 m*M*	0.05 m*M*	5–1000 m*M*	0.1 m*M*	10–100 m*M*	3 m*M*
2-Mercaptoethanol	0.01%	1 m*M*	1.0 *M*	0.1 m*M*	10–100 m*M*	10 m*M*
Thimerosal	0.01%	0.01%	0.01%	na	na	na
Chelators						
EDTA	10 m*M*	1 m*M*	100 m*M*	na	5–10 m*M*	30 m*M*
EGTA	na	1 m*M*	2 m*M*	na	na	na
Solvents						
DMSO	10%	10%	10%	na	na	20%
Ethanol	10%	10%	10%	na	na	na
Glycerol	10%	10%	10%	10%	10%	40%

Guanidine–HCl	4.0 *M*	0.1 *M*	3.5 *M*	na	na	na
Methanol	10%	10%	10%	na	na	na
PMSF	1 m*M*	1 m*M*	1 m*M*	na	na	na
Sucrose	40%	7.5%	10%	10%	10–500 m*M*	2 *M*
Urea	3.0 *M*	3.0 *M*	3.0 *M*	na	na	>1.0 *M*
Miscellaneous						
DNA	0.1 mg	0.2 mg	0.25 mg	na	50–100 μg/ml	1 μg

Values relate to the maximum concentration of interfering compound within the protein sample that does not result in significant loss in assay performance. The guide is an updated version of that prepared by Stoscheck to inform of any issues related to assay interference. Concentrations were obtained from product inserts and references (Bradford, 1976; Peterson, 1979; Smith *et al.*, 1985; Stoscheck, 1990), where there is not a consensus of values a range is given. Changing the protein-to-dye ratios, or formulation of many of the dye-based assays can alter the maximum concentration of compound permissible. Interfering compounds have been selected to represent those commonly encountered in protein purification and enzymology.

[a] na indicates the reagent was not tested. A blank indicates that the reagent is not compatible with the assay at the reagent concentrations analyzed. A figure preceded by (<) or (>) symbols indicates the tolerable limit is unknown but is respectively, less than or greater than the amount shown.

[b] Figures indicate the concentration in a 0.1-ml sample using a final reaction volume of 2.1 ml.

[c] Figures indicate the concentration in a 0.2-ml sample using a final reaction volume of 1.3 ml.

[d] Figures indicate the concentration in a 0.05-ml sample using a final reaction volume of 1.55 ml.

[e] Figures indicate interference concentrations with the CBQCA™ assay (You *et al.*, 1997) in a 90-μl sample using a final reaction volume of 100 μl.

[f] Figures indicate interference concentrations with the NanoOrange™ and Quant-iT™ assays ((Hammer and Nagel, 1986) and Quant-iT™ product insert) in a 40- and 20-μl sample (respectively) using a final reaction volume of 200 μl.

[g] Figures indicate the concentration of the chemical that does not produce an absorbance of 0.5 over water (Stoscheck, 1990).

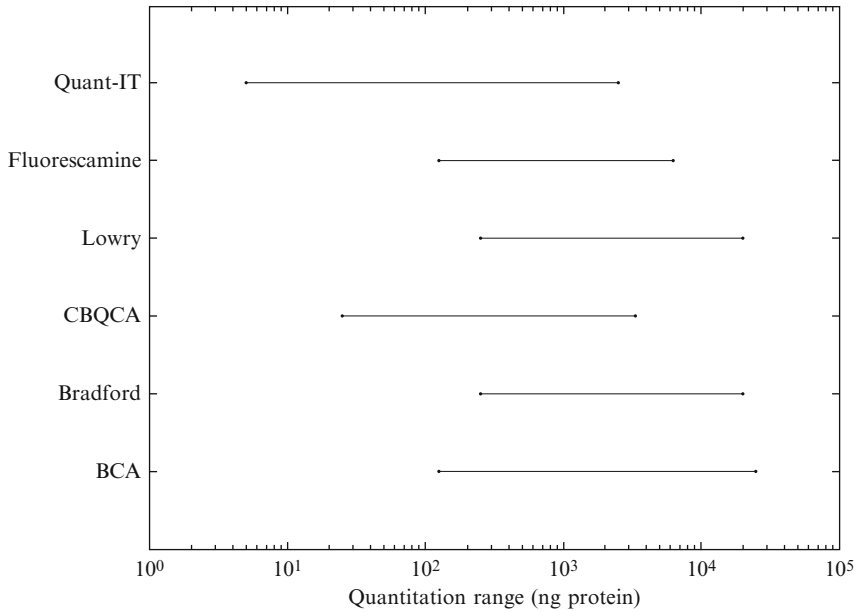

Figure 8.2 The markers designate the upper and lower values for the quantitation range of dye-based protein assay performed in a microplate format. The quantitation range was defined as the range of protein amounts (ng) that displayed good precision and did not show any deviation from the fitted response curve. Figure used with permission from Noble *et al.* (2007).

18 $M\Omega$ cm and a total organic carbon of below 6 ppb. All buffer preparations should be filtered using 0.2 μm filtration (Millipore, Sartorius) devices upon preparation to remove bacteria and fines. If precipitation occurs during storage, the reagent should be discarded, unless stated in the method.

3. Ultraviolet Absorption Spectroscopy

3.1. Ultraviolet absorbance at 280 nm (Range: 20–3000 μg)

Proteins display a characteristic ultraviolet (UV) absorption spectrum around 280 nm predominately from the aromatic amino acids tyrosine and tryptophan. If the primary sequence contains no or few of these amino acids then this method will give erroneous results. Quartz crystal cuvettes are routinely used for measurement as plastic materials can leach plasticizers, and are not UV transparent. Similarly, buffer components with strong UV absorbance such as some detergents especially Triton X-100 should be avoided (Table 8.1) and "blank" samples should be measured using the sample buffer solution but with

no protein present. UV absorbance is routinely used to give an estimate of protein concentration but if the molar extinction coefficient of the protein is known then the Beer–Lambert law can be used to accurately quantitate amount of protein by UV absorbance, assuming the protein is pure and contains no UV absorbing nonprotein components such as bound nucleotide cofactors, heme, or iron–sulfur centers.

Beer–Lambert (molar absorption coefficient):

$$A = a_{\mathrm{m}} c l \tag{8.1}$$

where a_{m} is the molar extinction coefficient, c the concentration of analyte, and l the path length in cm.

3.2. Method

For the measurement of a protein with unknown extinction coefficient, using a protein standard:

1. Add blank buffer to a clean quartz cuvette and use to zero the spectrophotometer.
2. Either using a fresh identical cuvette or replace the buffer with the sample, then measure the absorbance at 280 nm. If the signal is outside the linear range of the instrument (typically an absorbance greater than 2.0), then dilute the protein in buffer and remeasure.
3. After measurement of the sample remeasure the blank buffer to correct for any instrument drift.
4. Determine the unknown concentrations from the linear standard response.

3.3. Comments

The determination of the absorbance coefficient for a protein is discussed below but if a stock of the protein at known concentration is available then this can be used as a standard. Very rough estimates can be made from the relationship that if the cuvette has a path length of 1 cm, and the sample volume is 1 ml then concentration (mg/ml) = absorbance of protein at 280 nm.

Light scattering from either turbid protein samples or particles suspended in the sample with a comparable size to the incident wavelength (250–300 nm) can reduce the amount of light reaching the detector leading to an increase in apparent absorbance. Filtration using 0.2 μm filter units (that do not adsorb proteins), or centrifugation can be performed prior to analysis to reduce light scattering. Corrections for light scattering can be performed by measuring absorbance at lower energies (320, 325, 330, 335, 340, 345, and 350 nm), assuming the protein does not display significant absorbance at these wavelengths. A log–log plot of absorbance versus wavelength should

generate a linear response that can be extrapolated back to 280 nm, the resulting antilog of which will give the scattering contribution at this wavelength (Leach and Scheraga, 1960).

Nucleic acids absorb strongly at 280 nm and are a common contaminant of protein preparations. A pure protein preparation is estimated to give a ratio of A_{280} to A_{260} of 2.0 while, if nucleic acid is present, the protein concentration can be derived by the following formula (Groves *et al.*, 1968).

$$\text{Protein concentration (mg/ml)} = 1.55A_{280} - 0.76A_{260} \tag{8.2}$$

3.4. Ultraviolet absorbance at 205 nm (Range: 1–100 μg)

The peptide bond absorbs photons at a maximum wavelength below 210 nm. However, the broad absorption peak of the peptide bond allows measurements at longer wavelengths, which can have many practical advantages in terms of instrumentation and measurement accuracy. Due to interference from solvents and components of biological buffers, absorbance at 214 and 220 nm is often used as an alternative to measure proteins and peptides.

The large number of peptide bonds within proteins can make $\text{Abs}_{205\text{ nm}}$ measurements more sensitive and display less protein-to-protein variability than $\text{Abs}_{280\text{ nm}}$ measurements. Most proteins have extinction coefficients at $\text{Abs}_{205\text{ nm}}$ for a 1-mg/ml solution of between 30 and 35; however, an improved estimate can be obtained using Eq. (8.3) that takes into account variations in tryptophan and tyrosine content of the protein to be quantitated (Scopes, 1974). Absorbance at 205 nm is used to quantitate dilute solutions, or for short path length applications, for example, continuous measurement in column chromatography, or for analysis of peptides where there are few, if any aromatic amino acids.

$$\varepsilon_{205\text{ nm}}^{1\text{ mg/ml}} = 27.0 + 120 \times \left(\frac{A_{280\text{ nm}}}{A_{205\text{ nm}}}\right) \tag{8.3}$$

3.5. Calculation of the extinction coefficient

The extinction coefficient (ε) at a set wavelength describes the summation of all the photon absorbing species present within the molecule at a defined wavelength; the molar extinction coefficient is defined in Eq. (8.1). The extinction (absorption) coefficient is commonly expressed either in terms of molarity ($M^{-1}\,cm^{-1}$) or as a percentage of the mass $\varepsilon^{1\%}$ ($\%^{-1}\,cm^{-1}$), where $\varepsilon^{1\%}$ is defined as the absorbance value of a 1% protein solution.

Deviation from experimentally derived values for ε, and those derived by sequence data can be due to the influence of salts and buffers within the

protein sample. The absorbance spectra from various amino acids are environmentally sensitive; therefore, ε derived for a protein in a set buffer may not be the same for another buffer system if gross changes in pH (tyrosine ionization at pH 10.9), or solvent polarity (denaturing agents) occur.

To determine ε_{280}, the amino acid composition or sequence of the protein is required. From the protein sequence, ε_{280} can be calculated from first principles using a standard formula (Gill and von Hippel, 1989), which has been refined (Pace *et al.*, 1995). Such models use the absorption coefficients for specific amino acids (Trp, Tyr, and disulfide bond) to generate a good estimate of ε_{280} where these amino acids are in abundance. However, where there is a low abundance of these amino acids (e.g., insulin), the model can display deviations of up to 15% from that determined by physical methods (Pace *et al.*, 1995). Physical (empirical) methods to determine extinction coefficient include amino acid analysis (AAA) via acid hydrolysis and chromatographic separation of resulting amino acids (Sittampalam *et al.*, 1988) and Kjeldahl and gravimetric analysis (Kupke and Dorrier, 1978).

4. Dye-Based Protein Assays

Methods to prepare the established (nonproprietary) protein quantitation assays are described. These reagents can be economically prepared in bulk and stored for prolonged periods. The majority of such assays are available from commercial suppliers such as Sigma-Aldrich, Bio-Rad, Novagen, and Pierce. It should be noted that suppliers can have different preparations of such reagents and these can perform differently with specific proteins. The use of commercial reagents can improve the long-term repeatability and performance of the assay and for microplate-based assays is reported to reduce issues with dye precipitation after long-term storage of reagents (Stoscheck, 1990). The majority of the spectrophotometric protein quantitation methods described can be adapted to a microplate format (typically 96-well plate), we have highlighted where changes in the assay formulations are required.

4.1. Protein concentration standards

The ideal protein standard to use in a quantitative assay is the exact same protein in a matched matrix/solution that has been assigned using a higher order method, for example AAA (Sittampalam *et al.*, 1988) or gravimetric analysis (Blakeley and Zerner, 1975). Gravimetric analysis is prone to errors due to the extensive dialysis and drying to remove water and salts from commercial preparations. Prepared standards should be redissolved at a high concentration in water and stored at $-20\ ^{\circ}C$ for long-term storage.

In practice, there is not always a matched protein standard available; however, some commercially available standards may be suitable for use, the most common being BSA, bovine gamma globulins, or immunoglobulins (used for antibody quantitation). The use of a BSA standard is known to give misleading results in many assays, especially those methods that are sensitive to the protein sequence, that is where the signal is generated by specific amino acids (Fig. 8.3). Assays with a low protein sequence dependence will give better estimates when BSA calibration is compared to AAA assignment (Alterman *et al.*, 2003). AAA assignment quantitates the amount

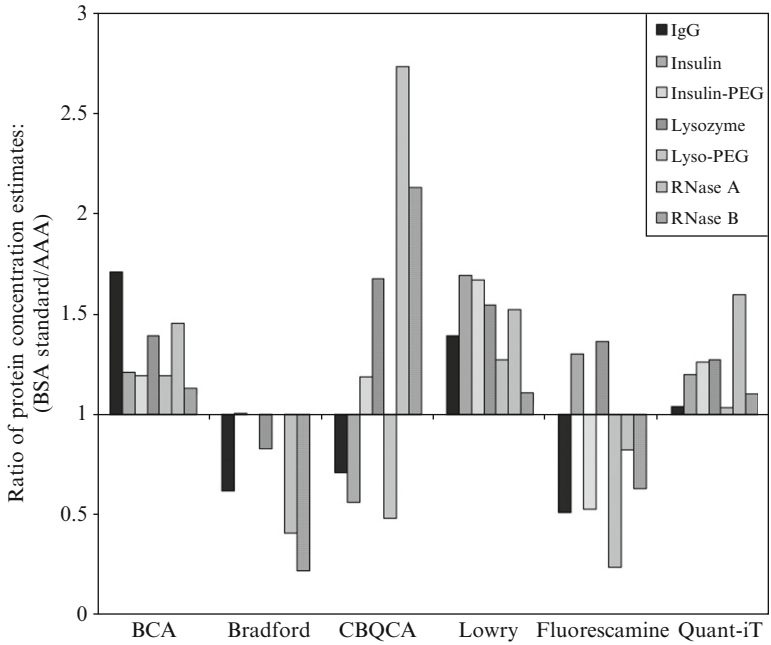

Figure 8.3 A comparison of the accuracy of the BSA standard in estimating the concentration of a protein using dye-based protein quantitation assays. AAA was used to determine the concentration of the model proteins; from these estimates a calibration curve for each protein was prepared using the dye-based assays. In the same plate, a calibration curve using the BSA standard (Pierce; concentration defined by manufacturer) was also prepared and the response of this was compared to that of the model proteins to see how well the BSA standard estimated the true concentration of the model proteins. The ''ratio of concentration estimations'' refers to the concentration of protein derived using the BSA standard when compared to the ''true'' value using AAA, where a ratio of 1 indicates the two methods gave the same value. The variation ''% CV'' associated with the dye-based protein concentration assays ranged from 2% to 8%, dependent on the assay. AAA concentration assignment typically displayed 5% CV values, dependent on the protein analyzed. Figure adapted with permission from Noble *et al.* (2007).

of specific amino acids present following protein hydrolysis and separation, using peptide sequence information the amount of target protein can then be calculated.

5. Coomassie Blue (Bradford) Protein Assay (Range: 1–50 μg)

The Bradford assay encompasses various preparations of the dye Coomassie Brilliant Blue G-250 used for protein quantitation purposes, and was first described by Bradford (1976). The basic mechanism of the assay is the binding of the dye at acidic pH to arginine, histidine, phenylalanine, tryptophan and tyrosine residues (de Moreno *et al.*, 1986), and hydrophobic interactions (Fountoulakis *et al.*, 1992). The exact mechanism is however still not fully understood (Sapan *et al.*, 1999). Upon binding protein, a metachromatic shift from 465 to 595 nm is observed due to stabilization of the anionic form of the dye. The majority of the observed signal is due to the interaction with arginine residues, resulting in the wide protein-to-protein variation characteristic of Bradford assays (Fig. 8.3).

5.1. Reagents

Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol and add 100 ml of 85% phosphoric acid while stirring continuously. When the dye has dissolved dilute to 1 l in water. The reagent is stable for up to a month at room temperature; however, for long-term storage keep at 4 °C, if precipitation occurs filter before use.

5.2. Procedure

1. Prepare standards in the range 100–1500 μg/ml in a Bradford-compatible buffer. For more dilute samples the sensitivity can be extended by increasing the ratio of sample to reagent volumes (Micro Bradford assay: 1–25 μg/ml). If the ratio of the sample to dye is too high, the pH of the reaction mixture could increase leading to higher background responses.
2. Add the standard and unknown samples to disposable cuvettes (plastic disposable cuvettes and microplates should be used as the dye sticks to various surfaces).
3. Allow the Bradford reagent to warm to room temperature. Add 1 ml of the dye solution to 25 μl of the protein sample, mix and incubate for 10 min at room temperature.

4. Measure the absorbance at 450 and 595 nm (for filter-based instruments a range from 570 to 610 nm can be used without significant loss of assay performance).
5. Plot either the 595 nm data or for improved precision at lower response values the ratio 595 nm/450 nm. The standard response curve can be fit to a polynomial response, from which unknown protein estimates can be calculated.

5.3. Comments

The advantages of the Bradford assay include the ease of use, sensitivity and low cost of the reagents. For microplate-based assays the reagent volumes can be decreased giving a total volume of 300 μl. Due to the path of the light source on the majority of microplate spectrophotometers, it is recommended to use commercial sources of Bradford reagent that are less predisposed to precipitation during prolonged storage.

We have observed significant variation in response between various commercial suppliers of Bradford preparations (Noble *et al.*, 2007). This appears to be most pronounced when analyzing low-molecular-weight proteins or peptides. Indeed the assay is reported to display a molecular weight cutoff "threshold"; requiring a certain number of residues for full signal development (de Moreno *et al.*, 1986). Changes in the formulation of the Bradford reagent are reported to change the response generated from specific proteins; therefore, care should be taken when comparing Bradford data from different suppliers or preparations (Chan *et al.*, 1995; Friedenauer and Berlet, 1989; Lopez *et al.*, 1993; Read and Northcote, 1981).

The Bradford assay is sensitive to interferences from various reagents detailed in Table 8.1 that include most ionic and nonionic detergents and glycosylated proteins. If precipitation of the reaction mixture occurs, for example hydrophobic or membrane proteins, the reaction can be supplemented with 1 *M* NaOH at 5–10% (v/v) to aid solubilization.

6. Lowry (Alkaline Copper Reduction Assays) (Range: 5–100 μg)

The Lowry assay (Lowry *et al.*, 1951) and other preparations with enhanced assay performance are based on a two-step procedure. Initially, the Biuret reaction involves the reduction of copper (Cu^{2+} to Cu^{+}) by proteins in alkaline solutions, followed by the enhancement stage, the reduction of the Folin–Ciocalteu reagent (phosphomolybdate and phosphotungstate) (Peterson, 1979) producing a characteristic blue color with absorbance maxima at 750 nm. The assay displays protein sequence

variation, as color development is due not only to the reduced copper–amide bond complex but also to tyrosine, tryptophan, and to a lesser extent cystine, cysteine, and histidine residues (Peterson, 1977; Wu *et al.*, 1978).

The Lowry assay has been modified to reduce its sensitivity to interfering agents, increase the dynamic range and increase the speed and resulting stability of the color formation (Peterson, 1979). There are many commercial sources of the modified Lowry assay (Roche, Pierce, Bio-Rad, and Sigma), but different preparations may not give equal responses when using the same standard, dilution buffer, or interfering compounds.

6.1. Reagents

6.1.1. Folin and Ciocalteu's reagent

The preparation of this reagent has been described (Lowry *et al.*, 1951); however, the solution can be obtained from commercial sources (Sigma). Mix 10 ml of Folin–Ciocalteu's Phenol reagent to 50 ml of water.

6.1.2. Copper sulfate reagent

100 mg $CuSO_4 \cdot 5H_2O$ and 200 mg of sodium tartrate dissolved in 50 ml of water. Dissolve 10 g of sodium carbonate into 50 ml of water, then pour slowly while mixing to the copper sulfate solution, prepare fresh daily.

6.1.3. Alkaline copper reagent

Mix one-part copper sulfate solution, one-part 5% SDS (w/v) and two-parts 3.2% sodium hydroxide (w/v). This solution can be stored at room temperature for up to 2 weeks, discard if a precipitate forms.

6.2. Procedure

1. To 1 ml of sample and protein standards $\sim$ 5–100 μg/ml, add 1 ml of the alkaline copper reagent, mix and allow to stand for 10 min.
2. Add 0.5 ml of Folin–Ciocalteu's reagent mix, vortex thoroughly and incubate for 30 min.
3. After incubation vortex again and measure the absorbance at 750 nm. Absorbance can be read from 650 to 750 nm depending on the availability of appropriate filters (microplate readers), or if the signal is too high, without significant loss in assay performance. Lowry is not an endpoint assay, so samples should be staggered to obtain more accurate estimates.
4. The response observed will be linear over a limited range of standards. Polynomial, exponential, and logarithmic models can be used to fit the data to extend the dynamic range of the response curve.

6.3. Comments

The Lowry method above can be adapted to a microplate format by reducing the volume of reactants added, resulting in a dynamic range ~50–500 μg/ml. The Lowry assay has been largely superseded by the BCA assay due to sensitivity, linearity, and improved methodology.

The Lowry protein assay is sensitive to many interfering compounds (Table 8.1), which may not generate a linear response (making extrapolations of interfering data complex). Formation of precipitates can occur with detergents, lipids, potassium ions, and sodium phosphate.

7. Bicinchoninic Acid (BCA) (Range: 0.2–50 μg)

The BCA assay replaces the Folin–Cioalteu's reagent as described for the Lowry method with Bicinchoninic acid that results in a protein assay with improved sensitivity and tolerance to interfering compounds (Smith *et al.*, 1985). The BCA reaction forms an intense purple complex with cuprous ions (Cu^{+}) resulting from the reaction of protein and alkaline Cu^{2+}. The residues that contribute to the reduction of Cu^{2+} include the cysteine, cystine, tryptophan, tyrosine, and the peptide bonds (Smith *et al.*, 1985). The chemical reaction is temperature dependent with different functional groups displaying a different reactivity at elevated temperatures, which result in less protein variability (Wiechelman *et al.*, 1988). At elevated temperatures (60 °C compared to 37 °C), more color formation is observed due to the higher reactivity of tryptophan, tyrosine, and peptide bonds.

Most of the commercial preparations are formulated close to the original preparation described by Smith *et al.* (1985), which is described in the following subsections. Variations have been employed to improve the sensitivity of the assay and can be obtained from commercial sources (Pierce, Novagen). The sample-to-working reagent ratio can be varied to maximize signal, or reduce assay interference, typically ratios of 8–20-fold excess of BCA working reagent are added to the protein sample.

7.1. Reagents

Reagent A: 1 g sodium bicinchoninate, 2 g Na_2CO_3, 0.16 g sodium tartrate, 0.4 g NaOH, and 0.95 g $NaHCO_3$, made up to 100 ml and the pH adjusted to 11.25 with either solid or concentrated NaOH.

Reagent B: 0.4 g $CuSO_4 \cdot 5H_2O$ dissolved in 10 ml water. Both reagent A and B are stable indefinitely at room temperature.

The working solution is prepared by mixing 100 parts of reagent A with two parts reagent B to form a green solution that is stable for up to a week.

7.2. Procedure

1. Cuvette analysis can be performed with 50–150 μl of protein and 3 ml of BCA working reagent, whereas microplate assay can use 25 μl of protein and 200 μl of BCA working reagent, that is a lower reagent to protein ratio.
2. Incubate the sample and standards ~5–250 μg/ml at either 37 or 60 °C for 30 min (longer incubations at 37 °C will improve protein-to-protein variability) and allow the sample to equilibrate to room temperature before reading. Microplates should be covered during incubation to avoid evaporation of the sample. For cuvette analysis at 37 °C, samples should be staggered to ensure equal incubation times.
3. Measure absorbance at 562 nm, for filter-based plate readers wavelengths in the range of 540–590 nm can be used instead without a significant loss in assay performance.
4. The BCA assay will produce a linear response over a wide concentration range; however, to extend the dynamic range of the data analysis a quadratic response can be used to model the data.

7.3. Comments

A microbased BCA assay can be used to improve the sensitivity of the procedure (1–25 μg/ml). The microbased assay uses a more concentrated working solution and can be prone to precipitation; again commercial sources of this modified BCA assay are available (Pierce). The BCA assay is sensitive to either copper chelators (e.g., EDTA) or reagents that can also reduce Cu^{2+} (e.g., DTT), a summary of the maximum tolerances can be found in Table 8.1.

8. Amine Derivatization (Range: 0.05–25 μg)

Amine-labeling "derivatization" using various fluorescent probes is a common technique to quantitate amino acid mixtures in AAA. The same technique can be used to quantitate proteins and peptides containing either lysine or a free N-terminus, both of which need to be accessible to the dye. Upon reaction with amines, the dyes display a large increase in fluorescence that for part of the dynamic range will generate a linear response with increasing protein concentration. Three dyes that have been used to quantitate proteins, or amino acids in a microplate format include *o*-phthalaldehyde (OPA) (Hammer and Nagel, 1986), Fluorescamine (Lorenzen and Kennedy, 1993), and 3-(4-carboxybenzoyl)quinoline-2-carboxyaldehyde (CBQCA™) (Asermely *et al.*, 1997; Bantan-Polak *et al.*, 2001; You *et al.*, 1997).

Fluorescamine reacts directly with the amine functional group, whereas OPA and CBQCA^{TM} require the addition of a thiol (2-mercaptoethanol) or cyanide (CBQCA^{TM}). A cuvette-based format is described for the OPA assay, which can be converted to a microplate format by adjusting the volume of reactants and NaOH.

8.1. Reagents

OPA stock: Dissolve 120 mg of *o*-phthalaldehyde (high purity grade from Sigma or Invitrogen) in methanol, then dilute to 100 ml in 1 *M* boric acid, pH 10.4 (pH adjusted with potassium hydroxide). Add 0.6 ml of polyoxyethylene (23) lauryl ether and mix. The stock is stable for 3 weeks at room temperature.

8.2. Procedure

1. At least 30 min before analysis, add 15 μl of 2-mercaptoethanol to 5 ml of OPA stock, this reagent is stable for a day. Protect all fluorescent samples and reactions from light at all times.
2. Protein standards (0.2–10 μg/ml) and unknown samples need to be adjusted to a pH between 8.0 and 10.5 before analysis. Mix 10 μl of test sample with 100 μl of OPA stock (supplemented with 2-mercaptoethanol) and incubate at room temperature for 15 min.
3. Add 3 ml of 0.5 N NaOH and mix.
4. Read fluorescence at excitation 340 nm and emission from 440 to 455 nm in a fluorescent cuvette.
5. The relationship between protein concentration and fluorescence should be linear over the dynamic range of the assay and can be used to estimate unknown samples.

8.3. Comments

All three dyes offer improved sensitivity and dynamic range when compared with absorbance-based protein quantitation assays. OPA is generally preferred over fluorescamine due to its enhanced solubility and stability in aqueous buffers.

The use of amine-derivatization agents for protein quantitation is limited as the assay displays a large protein-to-protein variability due to variation in the number of lysine residues in proteins, requiring the need for a "matched" standard. Assay interference from glycine and amine containing buffers, ammonium ions, and thiols common in many biological-buffering systems limit the application of such assays (Table 8.1). The reproducibility of the assay is dependent on the pH of the reaction, protein samples that

contain residual acids, for example from precipitation steps could reduce the rate of amine derivatization (You *et al.*, 1997).

A noncovalent amine reactive dye epicocconone can also be used for total protein assays in solution (Sigma), for which the mechanism has been reported (Bell and Karuso, 2003; Coghlan *et al.*, 2005).

9. Detergent-Based Fluorescent Detection (Range: 0.02–2 μg)

The development of fluorescent probes whose quantum yields are enhanced significantly when binding at the detergent–protein interface have been used to quantitate proteins within gels and in solution-based assays (Daban *et al.*, 1991; Jones *et al.*, 2003). Two commercial preparations of these assays are available NanoOrange™ and Quant-iT™ (Invitrogen); however, limited independent testing of the respective reagents prevents a full critical analysis. The NanoOrange™ assay is limited by the need to heat samples to 90 °C to denature the proteins thereby reducing the protein-to-protein variability (Jones *et al.*, 2003). Both assays are sensitive to detergents and high salt concentrations (Table 8.1), which presumably disrupt the protein-dye-detergent interface. The Quant-iT™ assay displays good sensitivity and dynamic range compared to other dye-based assays (Fig. 8.2), and a relatively low protein-to-protein variability (Fig. 8.3).

10. General Instructions

The choice of measurement format used will depend on the throughput, sensitivity, and precision required of the assay, and concerns about assay interferences that can be reduced by dilution in cuvette-based assays. From our experience, both in industry and academia, plate-based assays are replacing cuvette assays due to increases in throughput. For all spectrophotometric techniques, the instrument should be warmed up for 15 min prior to measurement and any calibration programs run before sample analysis. Samples and reagents should be equilibrated to room temperature before analysis to avoid condensation on optical surfaces.

10.1. Cuvettes

Traditionally, cuvettes have been used for the majority of spectrophotometric protein assays. Quartz cuvettes can be costly, therefore glass cuvettes are preferred; however, both of these may have to be washed between measurements to remove dye and adsorbed protein. Disposable plastic

cuvettes are available and can be used to increase the throughput where many samples have to be measured, or the reagent is prone to sticking to the cuvette surface, for example Bradford reagent. Staggering of sample analysis is especially important if the signal is not stable, or does not run to completion within the time frame of the assay, for example BCA or Lowry assays. The best precision is obtained from a two-beam instrument incorporating a reference cell to account for instrument drift. Replacing the cuvette in the holder between each measurement due to cleaning, or the use of disposable cuvettes can result in changes in alignment, resulting in significant changes in amount of light reaching the detector. This is especially important if low-volume cuvettes are being used where the transmission window is reduced in size. Care should also be taken with low-volume cuvettes to ensure the sample covers the entire transmission window.

Care should be taken when handling and cleaning cuvettes. Prevent fingerprints from contaminating the transmitting surfaces. Cuvettes should be washed with either water or an appropriate solvent between runs and dried using a stream of nitrogen gas. If smearing of the transmitting surface is observed, the cuvette can be rewashed in water, ethanol, and finally acetone, or removed using ethanol and lintless lens tissue. If protein deposition is a recurring problem, cuvettes can be washed overnight in nitric acid and thoroughly washed before use.

10.2. Microwell plates

The majority of protein assays have been adapted for use in microwell plates, typically 96-well plates to enhance speed, throughput and lower sample and reagent usage. Many of the commercial fluorescent assays are specifically designed for plate formats. The plate reader format also offers the advantage of being able to read multiple samples within a short period (typically 25 s) reducing potential timing differences in reactions that do not go to completion, or are unstable.

Protein UV measurements can be made in a plate format; however, the effective path length can be difficult to calculate due to meniscus formation for concentrated protein solutions (many commercial plate readers can estimate effective path-length and thereby improving protein quantitation calculations). Quartz 96-well plates tend to be expensive, difficult to clean and prone to scratches that can affect light transmission.

Care should be taken in the preparation of protein assays in plate formats. The use of lower volume samples (down to 5 μl for some assays) can increase the relative pipetting errors of high viscosity solutions. Well-to-well contamination should be avoided by using fresh pipette tips for each sample and reagent. Regular calibration of the instrument should be performed using either optical standards or solid phase fluorescent standard plates (Matech) to ensure equal transmission/light detection from all wells.

Many of the 96-well plates conform to a standard geometry; however, in our experience, it is worth analyzing plate geometry in the plate-reader, especially if a different plate supplier is used to ensure equal illumination, and detection for fluorescent-based measurements. Plate-based assays can also be more sensitive to sample precipitation (common in the Lowry and Bradford assays) when compared to cuvette-based assay due to the detection geometry.

Recently, spectrophotometers that can measure low microliter samples (typically 1–2 μl), without the need for a cuvette or microplates have become commercially available (Tecan and Thermo Scientific), further minimizing sample usage.

10.3. Interfering substrates

Interfering substances for many protein preparations will be variable from batch-to-batch and can be difficult to adequately control for when standards are formulated differently. The choice of assay used should take into account interfering contaminants in the protein preparation, either used as stabilizers or as a result of purification that cannot be replaced, or substituted with a suitable alternative, for example reducing agents or chelators. Inclusion of an interfering substance can be accommodated using a matched standard; however, this can result in loss of dynamic range and poor assay performance and is therefore not recommended. The concentration or amount of interfering substance that can be tolerated is often quoted with the assay instructions; however, this can be dependent on the formulation of the assay, the maximum tolerated concentrations are summarized in Table 8.1.

Interfering substances can be removed prior to concentration determination, however, this adds additional steps to the procedure and can often result in dilution, or incomplete recovery of the original sample leading to errors in the concentration estimate. Changes in sample recovery can be compensated for by comparing the recovery of the standard that has been subjected to interference removal steps.

Precipitation of protein followed by separation and resuspension probably offers the most accurate method to remove interfering substances where they cannot be avoided. Buffer components, detergents, and lipids can be removed by precipitating the protein with trichloroacetic acid (TCA), perchloric acid (PCA), or acetone (Olson and Markwell, 2007); however Triton X-100 can coprecipitate with TCA and PCA.

In addition to precipitation techniques, specific interferences can be removed through chemical treatment, for example reducing agents (iodoacetic acid treatment), lipids through chloroform extraction, volatility, or neutralization of strong acids/bases.

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REFERENCES

Alterman, M., Chin, D., Harris, R., Hunziker, P., Le, A., Linskens, S., Packman, L., and Schaller, J. AAARG2003 study: Quantitation of proteins by amino acid analysis and colorimetric assays. Poster available for download from www.abrf.org (http://www.abrf.org/ResearchGroups/AminoAcidAnalysis/EPosters/aaa2003_poster_print.pdf).

Asermely, K. E., Broomfield, C. A., Nowakowski, J., Courtney, B. C., and Adler, M. (1997). Identification of a recombinant synaptobrevin-thioredoxin fusion protein by capillary zone electrophoresis using laser-induced fluorescence detection. *J. Chromatogr. B Biomed. Sci. Appl.* **695,** 67–75.

Bantan-Polak, T., Kassai, M., and Grant, K. B. (2001). A comparison of fluorescamine and naphthalene-2, 3-dicarboxaldehyde fluorogenic reagents for microplate-based detection of amino acids. *Anal. Biochem.* **297,** 128–136.

Bell, P. J., and Karuso, P. (2003). Epicocconone, a novel fluorescent compound from the fungus epicoccumnigrum. *J. Am. Chem. Soc.* **125,** 9304–9305.

Blakeley, R. L., and Zerner, B. (1975). An accurate gravimetric determination of the concentration of pure proteins and the specific activity of enzymes. *Methods Enzymol.* **35,** 221–226.

Bradford, M. 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.

Burkitt, W. I., Pritchard, C., Arsene, C., Henrion, A., Bunk, D., and O'Connor, G. (2008). Toward Systeme International d'Unite-traceable protein quantification: From amino acids to proteins. *Anal. Biochem.* **376,** 242–251.

Chan, J. K., Thompson, J. W., and Gill, T. A. (1995). Quantitative determination of protamines by coomassie blue G assay. *Anal. Biochem.* **226,** 191–193.

Coghlan, D. R., Mackintosh, J. A., and Karuso, P. (2005). Mechanism of reversible fluorescent staining of protein with epicocconone. *Org. Lett.* **7,** 2401–2404.

Daban, J. R., Bartolome, S., and Samso, M. (1991). Use of the hydrophobic probe Nile red for the fluorescent staining of protein bands in sodium dodecyl sulfate-polyacrylamide gels. *Anal. Biochem.* **199,** 169–174.

de Moreno, M. R., Smith, J. F., and Smith, R. V. (1986). Mechanism studies of coomassie blue and silver staining of proteins. *J. Pharm. Sci.* **75,** 907–911.

Fountoulakis, M., Juranville, J. F., and Manneberg, M. (1992). Comparison of the Coomassie brilliant blue, bicinchoninic acid and Lowry quantitation assays, using non-glycosylated and glycosylated proteins. *J. Biochem. Biophys. Methods* **24,** 265–274.

Friedenauer, S., and Berlet, H. H. (1989). Sensitivity and variability of the Bradford protein assay in the presence of detergents. *Anal. Biochem.* **178,** 263–268.

Gill, S. C., and von Hippel, P. H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182,** 319–326.

Groves, W. E., Davis, F. C. Jr., and Sells, B. H. (1968). Spectrophotometric determination of microgram quantities of protein without nucleic acid interference. *Anal. Biochem.* **22,** 195–210.

Hammer, K. D., and Nagel, K. (1986). An automated fluorescence assay for subnanogram quantities of protein in the presence of interfering material. *Anal. Biochem.* **155,** 308–314.

Jones, L. J., Haugland, R. P., and Singer, V. L. (2003). Development and characterization of the NanoOrange protein quantitation assay: A fluorescence-based assay of proteins in solution. *Biotechniques* **34,** 850–854, 856, 858 *passim*.

Kupke, D. W., and Dorrier, T. E. (1978). Protein concentration measurements: The dry weight. *Methods Enzymol.* **48,** 155–162.

Leach, S. J., and Scheraga, H. A. (1960). Effect of light scattering on ultraviolet difference spectra. *J. Am. Chem. Soc.* **82,** 4790–4792.

Lopez, J. M., Imperial, S., Valderrama, R., and Navarro, S. (1993). An improved Bradford protein assay for collagen proteins. *Clin. Chim. Acta* **220,** 91–100.

Lorenzen, A., and Kennedy, S. W. (1993). A fluorescence-based protein assay for use with a microplate reader. *Anal. Biochem.* **214,** 346–348.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

Noble, J. E., Knight, A. E., Reason, A. J., Di Matola, A., and Bailey, M. J. (2007). A comparison of protein quantitation assays for biopharmaceutical applications. *Mol. Biotechnol.* **37,** 99–111.

Olson, B. J., and Markwell, J. (2007). Assays for determination of protein concentration. *Curr. Protoc. Protein Sci.* Chapter 3, Unit 3.4.

Ozols, J. (1990). Amino acid analysis. *Methods Enzymol.* **182,** 587–601.

Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995). How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4,** 2411–2423.

Peterson, G. L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83,** 346–356.

Peterson, G. L. (1979). Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. *Anal. Biochem.* **100,** 201–220.

Read, S. M., and Northcote, D. H. (1981). Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. *Anal. Biochem.* **116,** 53–64.

Sapan, C. V., Lundblad, R. L., and Price, N. C. (1999). Colorimetric protein assay techniques. *Biotechnol. Appl. Biochem.* **29**(Pt 2), 99–108.

Scopes, R. K. (1974). Measurement of protein by spectrophotometry at 205 nm. *Anal. Biochem.* **59,** 277–282.

Sittampalam, G. S., Ellis, R. M., Miner, D. J., Rickard, E. C., and Clodfelter, D. K. (1988). Evaluation of amino acid analysis as reference method to quantitate highly purified proteins. *J. Assoc. Off. Anal. Chem.* **71,** 833–838.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150,** 76–85.

Stoscheck, C. M. (1990). Quantitation of protein. *Methods Enzymol.* **182,** 50–68.

Wiechelman, K. J., Braun, R. D., and Fitzpatrick, J. D. (1988). Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal. Biochem.* **175,** 231–237.

Wu, A. M., Wu, J. C., and Herp, A. (1978). Polypeptide linkages and resulting structural features as powerful chromogenic factors in the Lowry phenol reaction. Studies on a glycoprotein containing no Lowry phenol-reactive amino acids and on its desialylated and deglycosylated products. *Biochem. J.* **175,** 47–51.

You, W. W., Haugland, R. P., Ryan, D. K., and Haugland, R. P. (1997). 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde, a reagent with broad dynamic range for the assay of proteins and lipoproteins in solution. *Anal. Biochem.* **244,** 277–282.