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The Bradford Method for Protein Quantitation

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1. Introduction

A rapid and accurate method for the estimation of protein concentration is essential in many fields of protein study. An assay originally described by Bradford ***(1)*** has become the preferred method for quantifying protein in many laboratories. This technique is simpler, faster, and more sensitive than the Lowry method. Moreover, when compared with the Lowry method, it is subject to less interference by common reagents and nonprotein components of biological samples (*see* **Note 1**).

The Bradford assay relies on the binding of the dye Coomassie Blue G250 to protein. Detailed studies indicate that the free dye can exist in four different ionic forms for which the pK_a values are 1.15, 1.82, and 12.4 ***(2)***. Of the three charged forms of the dye that predominate in the acidic assay reagent solution, the more cationic red and green forms have absorbance maxima at 470 nm and 650 nm, respectively. In contrast, the more anionic blue form of the dye, which binds to protein, has an absorbance maximum at 590 nm. Thus, the quantity of protein can be estimated by determining the amount of dye in the blue ionic form. This is usually achieved by measuring the absorbance of the solution at 595 nm (*see* **Note 2**).

The dye appears to bind most readily to arginyl and lysyl residues of proteins ***(3,4)***. This specificity can lead to variation in the response of the assay to different proteins, which is the main drawback of the method (*see* **Note 3**). The original Bradford assay shows large variation in response between different proteins ***(5–7)***. Several modifications to the method have been developed to overcome this problem (*see* **Note 4**). However, these changes generally result in a less robust assay that is often more susceptible to interference by other chemicals. Consequently, the original method devised by Bradford remains the most convenient and widely used formulation. Two types of assay are described here: the standard assay, which is suitable for measuring between 10 and 100 µg of protein, and the microassay, which detects between 1 and 10 µg of protein. The latter, although more sensitive, is also more prone to interference from other compounds because of the greater amount of sample relative to dye reagent in this form of the assay.

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2. Materials

1. Reagent: The assay reagent is made by dissolving 100 mg of Coomassie Blue G250 in 50 mL of 95% ethanol. The solution is then mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water (*see* **Note 5**).

   The reagent should be filtered through Whatman no. 1 filter paper and then stored in an amber bottle at room temperature. It is stable for several weeks. However, during this time dye may precipitate from solution and so the stored reagent should be filtered before use.
2. Protein standard (*see* **Note 6**). Bovine γ-globulin at a concentration of 1 mg/mL (100 µg/mL for the microassay) in distilled water is used as a stock solution. This should be stored frozen at –20ºC. Since the moisture content of solid protein may vary during storage, the precise concentration of protein in the standard solution should be determined from its absorbance at 280 nm. The absorbance of a 1 mg/mL solution of γ-globulin, in a 1-cm light path, is 1.35. The corresponding values for two alternative protein standards, bovine serum albumin and ovalbumin, are 0.66 and 0.75, respectively.
3. Plastic and glassware used in the assay should be absolutely clean and detergent free. Quartz (silica) spectrophotometer cuvettes should not be used, as the dye binds to this material. Traces of dye bound to glassware or plastic can be removed by rinsing with methanol or detergent solution.

3. Methods

3.1. Standard Assay Method

1. Pipet between 10 and 100 µg of protein in 100 µL total volume into a test tube. If the approximate sample concentration is unknown, assay a range of dilutions (1, 1:10, 1:100, 1:1000). Prepare duplicates of each sample.
2. For the calibration curve, pipet duplicate volumes of 10, 20, 40, 60, 80, and 100 µL of 1 mg/mL γ-globulin standard solution into test tubes, and make each up to 100 µL with distilled water. Pipet 100 µL of distilled water into a further tube to provide the reagent blank.
3. Add 5 mL of protein reagent to each tube and mix well by inversion or gentle vortex-mixing. Avoid foaming, which will lead to poor reproducibility.
4. Measure the A_{595} of the samples and standards against the reagent blank between 2 min and 1 h after mixing (*see* **Note 7**). The 100 µg standard should give an A_{595} value of about 0.4. The standard curve is not linear, and the precise absorbance varies depending on the age of the assay reagent. Consequently, it is essential to construct a calibration curve for each set of assays (*see* **Note 8**).

3.2. Microassay Method

This form of the assay is more sensitive to protein. Consequently, it is useful when the amount of the unknown protein is limited (*see also* **Note 9**).

1. Pipet duplicate samples containing between 1 and 10 µg in a total volume of 100 µL into 1.5-mL polyethylene microfuge tubes. If the approximate sample concentration is unknown, assay a range of dilutions (1, 1:10, 1:100, 1:1000).
2. For the calibration curve, pipet duplicate volumes of 10, 20, 40, 60, 80, and 100 µL of 100 µg/mL γ-globulin standard solution into microfuge tubes, and adjust the volume to 100 µL with water. Pipet 100 µL of distilled water into a tube for the reagent blank.
3. Add 1 mL of protein reagent to each tube and mix gently, but thoroughly.

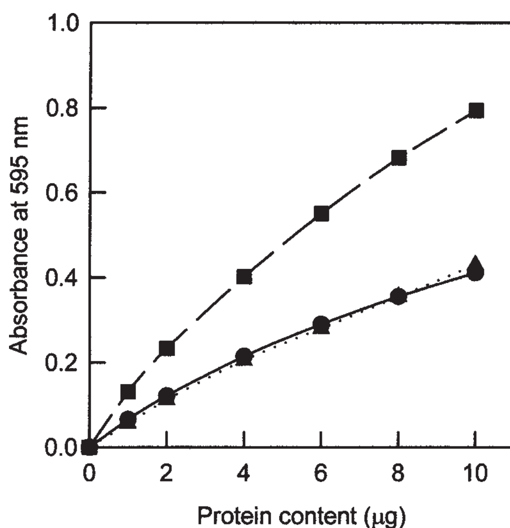

Fig. 1. Variation in the response of proteins in the Bradford assay. The extent of protein–dye complex formation was determined for bovine serum albumin (■), γ-globulin (●), and ovalbumin (▲) using the microassay. Each value is the mean of four determinations. For each set of measurements the standard error was <5% of the mean value. The data allow comparisons to be made between estimates of protein content obtained using these protein standards.

4. Measure the absorbance of each sample between 2 and 60 min after addition of the protein reagent. The A_{595} value of a sample containing 10 µg γ-globulin is 0.45. **Figure 1** shows the response of three common protein standards using the microassay method.

4. Notes

1. The Bradford assay is relatively free from interference by most commonly used biochemical reagents. However, a few chemicals may significantly alter the absorbance of the reagent blank or modify the response of proteins to the dye (**Table 1**). The materials that are most likely to cause problems in biological extracts are detergents and ampholytes ***(3,8)***. These can be removed from the sample solution by gel filtration, dialysis, or precipitation of protein with calcium phosphate ***(9,10)***. Alternatively, they can be included in the reagent blank and calibration standards at the same concentration as that found in the sample. The presence of base in the assay increases absorbance by shifting the equilibrium of the free dye toward the anionic form. This may present problems when measuring protein content in concentrated basic buffers ***(3)***. Guanidine hydrochloride and sodium ascorbate compete with dye for protein, leading to underestimation of the protein content ***(3)***.
2. Binding of protein to Coomassie Blue G250 may shift the absorbance maximum of the blue ionic form of the dye from 590 nm to 620 nm ***(2)***. It might, therefore, appear more sensible to measure the absorbance at the higher wavelength. However, at the usual pH of the assay, an appreciable proportion of the dye is in the green form (λ_{max} = 650 nm) which interferes with absorbance measurement of the dye–protein complex at 620 nm. Measurement at 595 nm represents the best compromise between maximizing the absorbence due to the dye–protein complex while minimizing that due to the green form of the free dye (***2–4***; but *see also* **Note 9**).

Table 1
Effects of Common Reagents on the Bradford Assay

	Absorbance at 600 nm	
Compound	Blank	5 mg Immunoglobulin
Control	0.005	0.264
0.02% SDS	0.003	0.250
0.1% SDS	0.042a	0.059a
0.1% Triton	0.000	0.278
0.5% Triton	0.051a	0.311a
1 *M* 2-Mercaptoethanol	0.006	0.273
1 *M* Sucrose	0.008	0.261
4 *M* Urea	0.008	0.261
4 *M* NaCl	–0.015	0.207a
Glycerol	0.014	0.238a
0.1 *M* HEPES, pH 7.0	0.003	0.268
0.1 *M* Tris, pH 7.5	–0.008	0.261
0.1 *M* Citrate, pH 5.0	0.015	0.249
10 m*M* EDTA	0.007	0.235a
1 *M* $(NH_4)_2SO_4$	0.002	0.269

Data were obtained by mixing 5 µL of sample with 5 µL of the specified compound before adding 200 µL of dye reagent.

[a]Measurements that differ from the control by more than 0.02 absorbance unit for blank values or more than 10% for the samples containing protein.

Data taken from **ref. *7***.

3. The dye does not bind to free arginine or lysine, or to peptides smaller than about 3000 Da ***(4,11)***. Many peptide hormones and other important bioactive peptides fall into the latter category, and the Bradford assay is not suitable for quantifying the amounts of such compounds.
4. The assay technique described here is subject to variation in sensitivity between individual proteins (*see* **Table 2**). Several modifications have been suggested that reduce this variability ***(5–7,12)***. Generally, these rely on increasing either the dye content or the pH of the solution. In one variation, adjusting the pH by adding NaOH to the reagent improves the sensitivity of the assay and greatly reduces the variation observed with different proteins ***(7)***. (This is presumably caused by an increase the proportion of free dye in the blue form, the ionic species that reacts with protein.) However, the optimum pH is critically dependent on the source and concentration of the dye (*see* ***Note 5***). Moreover, the modified assay is far more sensitive to interference from detergents in the sample.

   Particular care should be taken when measuring the protein content of membrane fractions. The conventional assay consistently underestimates the amount of protein in membrane-rich samples. Pretreatment of the samples with membrane-disrupting agents such as NaOH or detergents may reduce this problem, but the results should be treated with caution ***(13)***. A useful alternative is to precipitate protein from the sample using calcium phosphate and remove contaminating lipids (and other interfering substances, *see* **Note 1**) by washing with 80% ethanol ***(9,10)***.
5. The amount of soluble dye in Coomassie Blue G250 varies considerably between sources, and suppliers' figures for dye purity are not a reliable estimate of the Coomassie Blue G250 content ***(14)***. Generally, Serva Blue G is regarded to have the greatest dye content

Table 2
Comparison of the Response of Different Proteins in the Bradford Assay

	Relative absorbance	
Protein	Assay 1	Assay 2
Myelin basic protein	139	—
Histone	130	175
Cytochrome *c*	128	142
Bovine serum albumin	100	100
Insulin	89	—
Transferrin	82	—
Lysozyme	73	—
α-Chymotrypsinogen	55	—
Soybean trypsin inhibitor	52	23
Ovalbumin	49	23
γ-Globulin	48	55
β-Lactoglobulin A	20	—
Trypsin	18	15
Aprotinin	13	—
Gelatin	—	5
Gramicidin S	5	—

For each protein, the response is expressed relative to that of the same concentration of BSA. The data for assays 1 and 2 are recalculated from **refs.** ***5*** and ***7***, respectively.

and should be used in the modified assays discussed in **Note 4**. However, the quality of the dye is not critical for routine protein determination using the method described in this chapter. The data presented in **Fig. 1** were obtained using Coomassie Brilliant Blue G (C.I. 42655; product code B-0770, Sigma-Aldrich).

6. Whenever possible the protein used to construct the calibration curve should be the same as that being determined. Often this is impractical and the dye response of a sample is quantified relative to that of a "generic" protein. Bovine serum albumin (BSA) is commonly used as the protein standard because it is inexpensive and readily available in a pure form. The major argument for using this protein is that it allows the results to be compared directly with those of the many previous studies that have used bovine serum albumin as a standard. However, it suffers from the disadvantage of exhibiting an unusually large dye response in the Bradford assay, and thus, may underestimate the protein content of a sample. Increasingly, bovine γ-globulin is being promoted as a more suitable general standard, as the dye binding capacity of this protein is closer to the mean of those proteins that have been compared (**Table 2**). Because of the variation in response between different proteins, it is essential to specify the protein standard used when reporting measurements of protein amounts using the Bradford assay.
7. Generally, it is preferable to use a single new disposable polystyrene semimicrocuvette that is discarded after a series of absorbance measurements. Rinse the cuvette with reagent before use, zero the spectrophotometer on the reagent blank and then do not remove the cuvette from the machine. Replace the sample in the cuvette gently using a disposable polyethylene pipet.

8. The standard curve is nonlinear because of problems introduced by depletion of the amount of free dye. These problems can be avoided, and the linearity of the assay improved, by plotting the ratio of absorbances at 595 and 450 nm ***(15)***. If this approach is adopted, the absolute optical density of the free dye and dye–protein complex must be determined by measuring the absorbance of the mixture at each wavelength relative to that of a cuvette containing only water (and no dye reagent). As well as improving the linearity of the calibration curve, taking the ratio of the absorbances at the two wavelengths increases the accuracy and improves the sensitivity of the assay by up to 10-fold ***(15)***.
9. For routine measurement of the protein content of many samples the microassay may be adapted for use with a microplate reader ***(7,16)***. The total volume of the modified assay is limited to 210 µL by reducing the volume of each component. Ensure effective mixing of the assay components by pipetting up to 10 µL of the protein sample into each well before adding 200 µL of the dye reagent. If a wavelength of 595 nm cannot be selected on the microplate reader, absorbance may be measured at any wavelength between 570 nm and 610 nm. However, absorbance measurements at wavelengths other than 595 nm will decrease the sensitivity of response and may increase the minimum detection limit of the protocol.
10. For studies on the use of the Bradford assay in analyzing glycoproteins, *see* **Note 9** in Chapter 3.

References

1. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72,** 248–254.
2. Chial, H. J., Thompson, H. B., and Splittgerber, A. G. (1993) A spectral study of the charge forms of Coomassie Blue G. *Analyt. Biochem.* **209,** 258–266.
3. Compton, S. J. and Jones, C. G. (1985) Mechanism of dye response and interference in the Bradford protein assay. *Analyt. Biochem.* **151,** 369–374.
4. Congdon, R. W., Muth, G. W., and Splittgerber, A. G. (1993) The binding interaction of Coomassie Blue with proteins. *Analyt. Biochem.* **213,** 407–413.
5. Friendenauer, S. and Berlet, H. H. (1989) Sensitivity and variability of the Bradford protein assay in the presence of detergents. *Analyt. Biochem.* **178,** 263–268.
6. Reade, 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. *Analyt. Biochem.* **116,** 53–64.
7. Stoscheck, C. M. (1990) Increased uniformity in the response of the Coomassie Blue protein assay to different proteins. *Analyt. Biochem.* **184,** 111–116.
8. Spector, T. (1978) Refinement of the Coomassie Blue method of protein quantitation. A simple and linear spectrophotometric assay for <0.5 to 50 µg of protein. *Analyt. Biochem.* **86,** 142–146.
9. Pande, S. V. and Murthy, M. S. R. (1994) A modified micro-Bradford procedure for elimination of interference from sodium dodecyl sulfate, other detergents, and lipids. *Analyt. Biochem.* **220,** 424–426.
10. Zuo, S.-S. and Lundahl, P. (2000) A micro-Bradford membrane protein assay. *Analyt. Biochem.* **284,** 162–164.
11. Sedmak, J. J. and Grossberg, S. E. (1977) A rapid, sensitive and versatile assay for protein using Coomassie Brilliant Blue G250. *Analyt. Biochem.* **79,** 544–552.
12. Peterson, G. L. (1983) Coomassie blue dye binding protein quantitation method, in *Methods in Enzymology,* vol. 91 (Hirs, C. H. W. and Timasheff, S. N., eds.), Academic Press, New York.

13. Kirazov, L. P., Venkov, L. G. and Kirazov, E. P. (1993) Comparison of the Lowry and the Bradford protein assays as applied for protein estimation of membrane-containing fractions. *Analyt. Biochem.* **208,** 44–48.
14. Wilson, C. M. (1979) Studies and critique of amido black 10B, Coomassie Blue R and Fast Green FCF as stains for proteins after polyacrylamide gel electrophoresis. *Analyt. Biochem.* **96,** 263–278.
15. Zor, T. and Selinger, Z. (1996) Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Analyt. Biochem.* **236,** 302–308.
16. Redinbaugh, M. G. and Campbell, W. H. (1985) Adaptation of the dye-binding protein assay to microtiter plates. *Analyt. Biochem.* **147,** 144–147.