

Determination of Protein Concentration Using Bradford Microplate Protein Quantification Assay

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Abstract

Background: Bradford protein assay is popular due to its ease of performance and relative sensitivity. Many researchers and laboratories in Iran use standard assay of Bradford by cuvette. No commercial kit was available for Bradford microplate assay in Iran. Meanwhile, imported Bradford commercial kits are very expensive and have a long delivery time in Iran. Till now no study or document on Bradford microplate protein quantification assay was reported in Iran, so this study aimed to design and carried out this assay.

Methods: In the current study, antigen B of *hydatid* cyst fluid was used as sample and the assay was performed in microplate wells. The absorbance values were measured at 595 nm and standard curve was generated by Microsoft Office Excel software. The protein concentration of sample was calculated using the equation of the standard curve.

Results: Average protein concentration of the sample was 1175 µg/ml. The total time needed for reading of absorbance was two minutes approximately.

Conclusion: Bradford microplate protein assay is a fast and suitable method. This method could be replacing the time consuming method with cuvette. In addition, if this assay produces as a low price kit it could have many benefits for students and laboratories that need to determine protein concentration by Bradford assay.

Keywords: Analysis; Proteins; Laboratories; Biochemistry; Biological assay

Introduction

Proteins found in all biological systems, from prokaryotes to eukaryotes (1). Proteins are the most abundant class of biomolecules since they represent over 50% of the dry weight of cells. The measurement of protein concentration in an aqueous sample is an important assay in biochemistry research, development labs for applications ranging from enzymatic studies to providing data for biopharmaceutical lot release and other fields of protein study (1, 2). Various platforms and methods are available to quantitate proteins; one of them is dye-binding assays (1). In comparison to other methods, dye-binding assays can be run at a high throughput, using inexpensive reagents with equipment found in the majority of biochemical laboratories (1, 3). The Bradford assay was first described by Bradford and become the preferred method for quantifying protein in many laboratories (4). The basic mechanism of the assay is the binding

of coomassie brilliant blue G-250 dye at acidic pH to basic amino acid residues such as *arginine*, *histidine*, *phenylalanine*, *tryptophan*, *tyrosine* residues, and hydrophobic interactions in proteins which results in a color change to blue (1). The advantages of the Bradford assay include the ease of use, relative sensitivity, low cost of the reagents and low interference by other substances (4, 5). The Bradford assay can be performed in two different formats, cuvette assay and microplate assay (6). Usually standard assay procedure used 5 ml of Bradford reagent and 100 - 300 µl of each sample or standard which mixed in a cuvette (6, 7). The standard assay procedure with cuvette need more reagents and time to carry out than microplate assay. Meanwhile, in Iran most laboratories have used standard assay procedure with cuvette which has disadvantages such as long procedure time in cases that the number of samples and standards are very large. Till now, no document was available of using microplate

assay in Iran. Moreover, microplate assay kit was not available in Iran easily, due to high price of imported commercial kits and long delivery time. For these reasons the aim of this study was determination of protein concentration using Bradford microplate protein quantification assay.

Methods

Preparation of blank, standards and sample

In the current study, phosphate buffer was used as diluent. The blank consists of Phosphate buffer with

no protein. A series of protein standards consists of a known concentration of Bovine serum albumin (BSA, A8806 Sigma), prepared from 1mg/ml BSA source solution in the range 50-1000 $\mu\text{g/ml}$ in phosphate buffer in 0.5 ml tube (Table 1, Table 2). Antigen B (Ag B) of sheep *hydatid* cyst fluid was prepared by *oriol* method (8, 9) (Figure1). Ag B was used as sample with unknown concentration. The sample was diluted and prepared in 0.5 ml tube (Table 2). All blank, standards and sample prepared in duplicate (Figure 2).



Figure 1. Preparation of antigen B solution (as sample with unknown concentration of proteins); collection of *hydatid* cyst fluid from sheep liver *hydatid* cysts (1 and 2), separation of the larger particles of *hydatid* cyst fluid after centrifuge of 3000 g for 30 min (3), dialyze of *hydatid* cyst fluid against 5 mM acetate buffer (4), separation of antigens and globulins with centrifuge at 13000 g for 30 minutes. After that, the supernatant was discarded (5), dissolved pellet in phosphate buffer (6), sample after addition of ammonium sulfate (A4915-Sigma) on a magnetic stirrer for separation of immunoglobulin. After this stage supernatant collected by centrifuge at 3000 g for 30 minutes (7), sample in boiled water bath (memmert) for 15 min (Ag B is heat stable), after that supernatant containing Ag B separated by centrifuge at 13000 g for 30 min (8), the Ag B solution was filtered by a 0.2 μm syringe filter (Jet biofill), then antigen B solution was stored at -20°C until use (9) (References for these descriptions: 8, 9). (Pictures are original).

Preparation of assay reagent

The Bradford reagent (Figure 2) was prepared according to follow protocol: 100 mg coomassie brilliant blue G-250 (S D Fine Chem Limited), was dissolved in 50 ml 95% ethanol, then 100 ml 85% phosphoric acid (Merck Millipore, 100573) was added. Finally, the solution was diluted to one liter with distilled water (6, 10).

Assay procedure

The assay was performed using 50 μ l of each protein standard or unknown sample and 200 μ l of dye reagent in microplate wells (Figure 2). The content was mixed with plate shaker (Wise Cube, WIS-20) for 60 seconds (3, 6). The absorbance values of all the samples and controls were measured after five minutes incubation at room temperature at 595 nm by ELISA reader (Thermo Scientific Varioskan Flash Multimode Reader) (11).

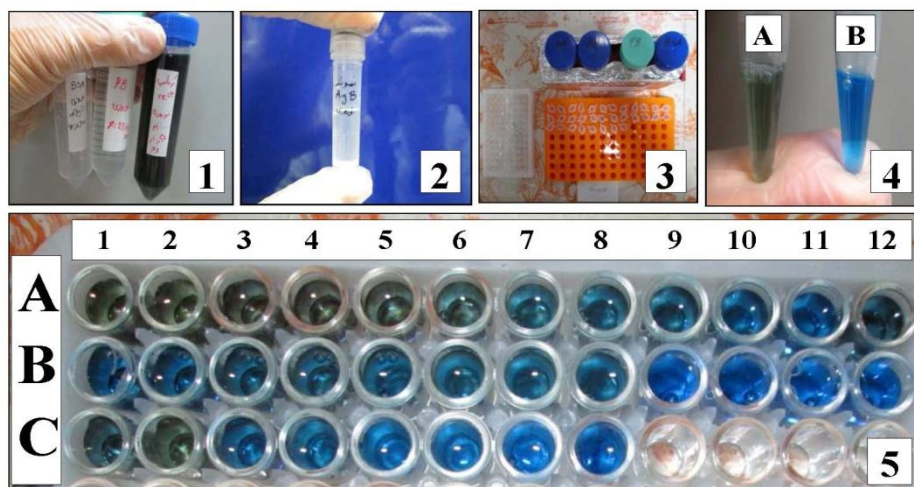


Figure 2. Materials and the reactions in Bradford microplate protein assay; Standard source, phosphate buffer and reagent (1), sample (Ag B) (2), preparation of blank, standards and sample dilutions in 0.5 ml tube (3), the reaction of assay reagent and BSA standard (assay reagent (A), the mixture of assay reagent with BSA standard (B))(4), the dye reaction of blank, BSA standards and sample dilutions (codes are according to Table 1 and Table 2) (5).

Standard curve

The average absorbance at 595 nm for blank (0.651) was subtracted from the absorbance at 595 nm for all other individual standard and sample dilutions (Table 1, Table 2) (12, 13). The standard curve and the equation of the calibration curve ($y = 0.002x + 0.014$) were generated by plotting the average blank corrected 595 nm measurement for each standard versus its concentration (μ g in 250 μ L) using Microsoft Office Excel by following the steps that shown in Figure 3 (6). The protein concentration of sample calculated using the below formula:

$$\text{Average protein concentration } (\mu\text{g in } 250 \mu\text{l}) = (\text{Absorbance} - 0.014) \div 0.002$$

Results

Average net absorbance at 595 nm (after subtraction of blank absorbance (0.651)) for eleven standards were included 0.048, 0.064, 0.117, 0.167, 0.211, 0.265, 0.301, 0.343, 0.401, 0.461 and 0.503 from 3A to 12 B codes respectively (Table 1 and Figure 4).

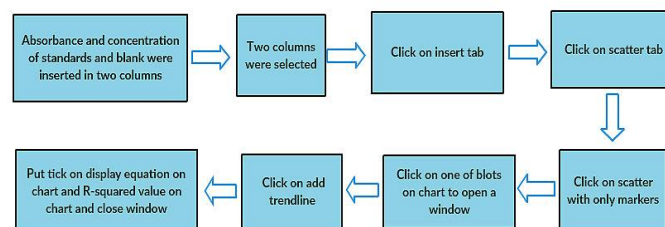


Figure 3. The steps that were carried out for the creation of BSA standard curve in Microsoft Office Excel software.

Table 1. Standards preparation scheme and resulted absorbance; each standard was prepared in duplicate

| Standard Code | Volume (μ l) of BSA stock solution | Volume (μ l) of Phosphate buffer | Volume (μ l) of transfer to microplate well | Volume (μ l) of reagent | Average BSA concentration (μ g in 250 μ l) | Average BSA concentration (μ g/ml) (dilution factor: five) | Average net absorbance at 595 nm (after subtraction of blank absorbance (0.651)) |
|---------------|---|---------------------------------------|--|------------------------------|---|---|--|
| 1A | 0 | 200 | 50 | 200 | | | |
| 2A | 0 | 200 | 50 | 200 | 0 | 0 | 0 |
| 3A | 10 | 190 | 50 | 200 | | | |
| 4A | 10 | 190 | 50 | 200 | 10 | 50 | 0.048 |
| 5A | 20 | 180 | 50 | 200 | | | |
| 6A | 20 | 180 | 50 | 200 | 20 | 100 | 0.064 |
| 7A | 40 | 160 | 50 | 200 | | | |
| 8A | 40 | 160 | 50 | 200 | 40 | 200 | 0.117 |
| 9A | 60 | 140 | 50 | 200 | | | |
| 10A | 60 | 140 | 50 | 200 | 60 | 300 | 0.167 |
| 11A | 80 | 120 | 50 | 200 | | | |
| 12A | 80 | 120 | 50 | 200 | 80 | 400 | 0.211 |
| 1B | 100 | 100 | 50 | 200 | | | |
| 2B | 100 | 100 | 50 | 200 | 100 | 500 | 0.265 |
| 3B | 120 | 80 | 50 | 200 | | | |
| 4B | 120 | 80 | 50 | 200 | 120 | 600 | 0.301 |
| 5B | 140 | 60 | 50 | 200 | | | |
| 6B | 140 | 60 | 50 | 200 | 140 | 700 | 0.343 |
| 7B | 160 | 40 | 50 | 200 | | | |
| 8B | 160 | 40 | 50 | 200 | 160 | 800 | 0.401 |
| 9B | 180 | 20 | 50 | 200 | | | |
| 10B | 180 | 20 | 50 | 200 | 180 | 900 | 0.461 |
| 11B | 200 | 0 | 50 | 200 | | | |
| 12B | 200 | 0 | 50 | 200 | 200 | 1000 | 0.503 |

Average net absorbance at 595 nm for 4 sample dilutions were consisted of 0.045, 0.134, 0.236, 0.484 from 1 C to 8 C codes respectively (Table 2). Average protein concentrations (μ g/ml) with the dilution factor of five in each sample dilution were included 77.5, 300, 555 and 1175 μ g/ml respectively.

So the average protein concentration (Antigen B) for sample in this study was 1175 μ g/ml. The total time for the reading of absorbance of all samples, standards and blank (thirty two well of microplate) was two minutes approximately.

Table 2. Sample preparation scheme and resulted absorbance; each diluted sample was prepared in duplicate

| Sample code | Volume (μ l) of sample solution | Volume (μ l) of Phosphate buffer | Volume (μ l) of transfer to microplate well | Volume (μ l) of reagent | Average net absorbance at 595 nm (after subtraction of blank absorbance (0.651)) | Average protein concentration (μ g in 250 μ l) | Average protein concentration (μ g/ml) (dilution factor: five) |
|-------------|--------------------------------------|---------------------------------------|--|------------------------------|--|---|---|
| 1C | 10 | 190 | 50 | 200 | | | |
| 2C | 10 | 190 | 50 | 200 | 0.045 | 15.5 | 77.5 |
| 3C | 60 | 140 | 50 | 200 | | | |
| 4C | 60 | 140 | 50 | 200 | 0.134 | 60 | 300 |
| 5C | 100 | 100 | 50 | 200 | | | |
| 6C | 100 | 100 | 50 | 200 | 0.236 | 111 | 555 |
| 7C | 200 | 0 | 50 | 200 | | | |
| 8C | 200 | 0 | 50 | 200 | 0.484 | 235 | 1175 |

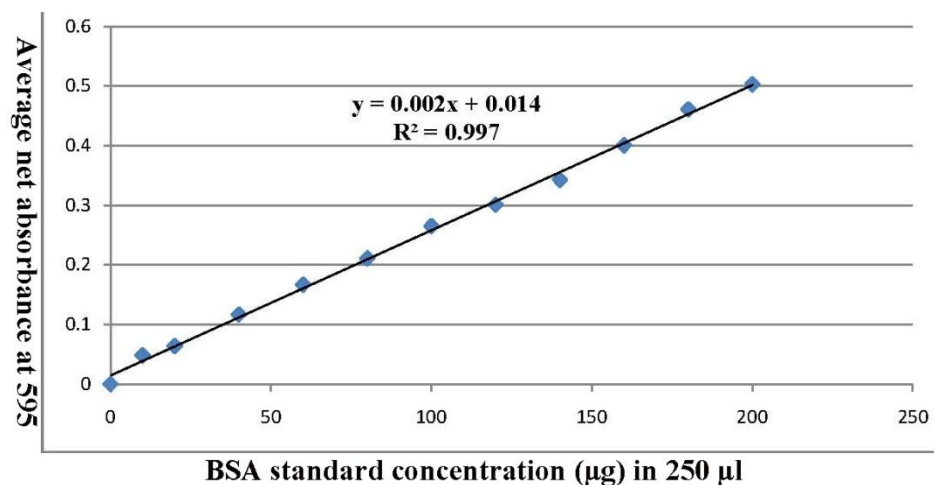


Figure 4. BSA standard curve; in equation (x) stand for sample concentration and (y) stand for average net absorbance

Discussion

Quantitation of total protein content is a measurement common to many applications in basic science and clinical research. The basis for this assay is the binding of coomassie brilliant blue G-250 to protein with a resultant shift in the absorbance maximum from 465 to 595 nm. Absorbance at 595 nm is used to quantitate protein content (14). The present study was first document in Iran which describes the Bradford microplate assay. The assay, which was described in the current study, has been used for years in the world. One of the problems that always exist in this assay is linearization of the standard curve. The absorbance in the Bradford assay varies from run to run depending on the batch of reagent used, the time between mixing and reading, and which reader is used. Therefore, it is best mixing the reagent in the standard wells at the same time as in unknown wells (15). Commercial kits for Bradford assay has produced by some companies such as Amresco (16), Thermo Scientific (11), Lamda Biotech (17), Koma biotech (18), Sigma-Aldrich (19), Serva (20), Bio-Rad (21), and Bio-world (22). Nevertheless, in Iran access to imported Bradford kits was not easy, due to sanctions and financial problems. In addition, the Bradford microplate

protein assay does not produce in Iran. In recent years, many studies on different aspects of Bradford assay by researchers such as Wenrich et al (23), Qian et al. (24), Gordon et al. (3), Carlsson et al. (25), Silvério et al. (26), Aminian et al. (27), Whiffen et al. (28) has conducted that most of them focused on the interference of chemicals with Bradford assay. The Bradford assay, is widely used because of its rapid and convenient protocol as well as its relative sensitivity (5). In comparison, with other dye binding methods, this technique is simpler, faster, and more sensitive than the Lowry method (6). Moreover, when compared with the Lowry method, it is subject to less interference by common reagents and non protein components of biological samples (6). In Bradford assay, unlike Bicinchoninic acid assay, reducing agents and metal chelators at low concentration do not cause interference (2, 1). Microplate assay is faster than standard assay with cuvette because absorbance of all standards, blank and samples measured simultaneously and this take approximately two minutes while for standard cuvette protocol more reagents and time needed as cuvette spectrophotometer in a specific time only capable of carried out performance with one cuvette. Bradford assay similar to any methods have

some limitations and disadvantages (1, 6), for example dye does not bind to free *arginine, lysine* or to peptides smaller than about 3000 Dalton (6). Many peptide hormones and other important bioactive peptides fall into the latter category, and the Bradford assay is not suitable for quantifying amounts of such compounds (6, 7). In conclusion production of the low price Bradford assay kit is possible in Iran, especially for university students who need it and don't afford to buy all materials separately or an imported kit.

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Conflicts of interest

The authors declare that there are no conflicts of interest related to the current article.

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