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Application Note

Protein Quantitation using Fluorescence

Total protein content is a measurement common to many applications in basic science and clinical research. Although several colorimetric techniques are available, they suffer several limitations dependent on the method. Recently, fluorescent techniques have become available that eliminate many of the problems associated with the traditional methods to measure total protein content. Here we describe a fluorescent method to quantitate total protein using the BioTek FL600 fluorescence microplate reader.

Introduction

Quantitation of total protein content is a measurement common to many applications in basic science and clinical research. Most biochemical studies that involve the measurement of a biological activity require the normalization of that activity to the protein content. The specific activity of a particular enzymatic activity is of particular importance when proteins are being purified or different samples are being compared. Regardless of the method of protein determination, laboratories requiring high throughput have often adapted the described protocol to a 96-well microplate based format. Here we describe a fluorescent method to quantitate total protein in the 96-well microplate based format using the BioTek FL600 fluorescence microplate reader.

Over the years, several methods to quantify protein have been developed. The most utilized methods to assay total protein content rely on the reduction of copper in the presence of a chromogenic reagent (1, 2). These methods work well, but are subject to interference by many compounds commonly used in protein purification, namely detergents and reducing agents. Because these methods rely on the presence of readily oxidizable amino acids such as tyrosine, cysteine, and tryptophan there is also a large variation in response from proteins with differing amino acid content. Simple absorbance measurements of protein solutions at 280 nm (A_{280}) are also subject to protein to protein variability, as well as interference from any contaminating nucleic acids. As a result of these difficulties, several dye-binding protein assays have been developed, the most commonly used being the method described by Bradford (3). This assay, which depends on the conversion of Coomassie Brilliant Blue G-250 to its blue form upon binding to protein, is subject to the formation of aggregates leading to a loss of signal over time. However, the binding of the compound NanoOrange™ with protein, which results in the formation of a fluorescent moiety, does not lead to aggregation or loss of signal over time.

Materials and Methods

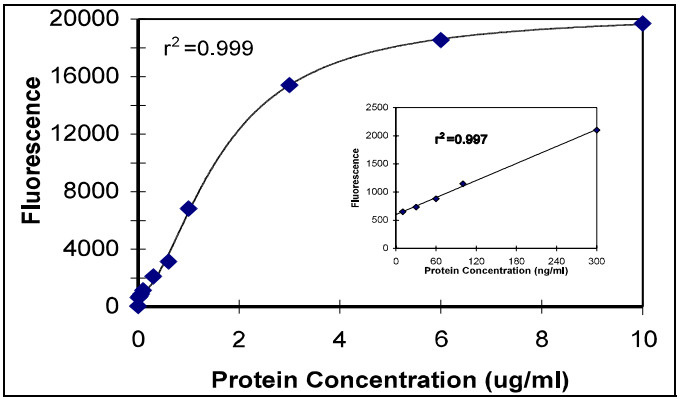
A NanoOrange™ Protein Quantitation Kit, catalogue number N-6666, was purchased from Molecular Probes (Eugene, Oregon). The 96 well black microplates with clear bottoms, catalogue number 3603, were purchased form Costar, (Cambridge, Massachusetts).

A series of dilutions ranging from 0.0 to 10 μg/ml of Bovine Serum Albumin (BSA) were made using 1X NanoOrange working solution as the diluent. Working NanoOrange solution was

prepared by diluting concentrated NanoOrange stock solution, provided in the NanoOrange kit, 1:10 with distilled water according to the kit instructions (2). After dilution of the protein sample, the protein solution was incubated for 10 minutes at 95°C. After heating, the solution was allowed to cool to room temperature and 200 μl aliquots were pipetted into microplate wells in replicates of eight. Fluorescence was determined using a BioTek Instruments FL600 fluorescent plate reader with a 485 nm, 20 nm bandwidth, excitation filter and a 530 nm, 25 nm bandwidth emission filter. The sensitivity setting was at 44 and the data collected from the bottom using static sampling with a 0.35 second delay, 100 reads per well. Although in these experiments the plates were read immediately, if they remained sealed and protected from light, the reaction was found to be stable for several hours.

Results

The fluorescence intensity was determined for BSA protein concentrations ranging from 0.0 to 10 μg/ml. Over this range the fluorescent intensity increased in a hyperbolic fashion. Using KinetiCalc data reduction software (BioTek Instruments), a 4-parameter non linear equation describing the standard curve can be generated. When the linearity of the reaction is examined, a least means squared straight line can be utilized for DNA concentrations up to 300 ng/ml with very high confidence.



Linearity of the Assay. Concentration curve from 0.0 to 10 μg/ml of BSA with 4-parameter regression analysis. Insert figure depicts the data points for the lowest protein concentrations (10 to 300 ng/ml). Filled diamonds (◆) represent the mean values of eight determinations at each concentration.

The average coefficient of variance for the standards was 7.5% with the greatest variation taking place primarily at the highest protein concentrations tested (data not shown). Although the curve begins to plateau at a protein concentrations of 5 μg/ml, determinations can be made with a high level of confidence (r^2 = 0.999). Determinations in the middle portion of the curve offer the greatest degree of accuracy with a 4-parameter logistic fit due to the greater change in signal verses change in protein concentration. Routine dilution of each sample would be expected to provide determinations at an appropriate concentration. In terms of sensitivity, the reaction was found to be sensitive to the nanogram levels, with fluorescent intensity values for 10 ng/ml being statistically different from the 0 μg control ($P<0.002$). The coefficient of determination (r^2=0.997) value indicates that concentration determinations can be made with a high degree of confidence in these very low concentrations.

This fluorescent total protein assay in conjunction with the BioTek FL600 offers several advantages. Because the SDS in the working solution maintains the denatured condition of the protein following heat denaturation, samples can be read hours later with no loss of sensitivity if

samples have been protected from light. In addition, the BioTek FL600 exceeded the kit manufacturer's reported detection limit by a factor of 10. This provides the investigator with greater sensitivity in a microplate format than would normally be expected. Like most assays that are read in microplates, the ability to read all of the samples simultaneously greatly reduces the manual labor required to obtain the data. The microplate format also lends itself to "off the shelf" automation for laboratories with high volume requirements. The smaller reaction volumes in microplates will lead to lower per assay costs by reducing the amount of expensive reagents necessary to perform the assay.

References

(1) Lowry, O.H., et. al. (1951) *Journal of. Biological Chemistry* **193:**265-275.
(2) Smith, P.K., et.el.. (1985). *Analytical Biochemistry* **150:**76-85.
(3) Bradford, M.M. (1976). *Analytical Biochemistry* **72:**248-254.
(4) NanoOrange Protein Quantitation Kit Instructions Molecular Probes, Inc. Eugene Oregon.

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