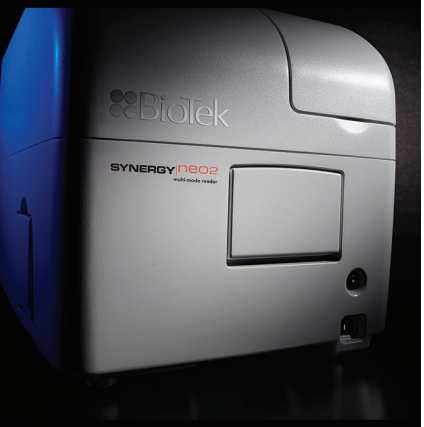
Protein Quantitation using NanoOrange® Fluorescence

Using Synergy™ Neo2 Multi-Mode Reader to Quantify Total Protein

Paul Held, Ph. D, Laboratory Manager, Applications Department, BioTek Instruments, Inc., Winooski, VT USA



Key Words:

NanoOrange®
Protein Quantitation
Total Protein

Abstract

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. 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 BioTek's Synergy™ Neo2 Multi-Mode 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 fluorescence method to quantify total protein in the 96-well microplate based format using BioTek's Synergy Neo2 Multi-Mode Reader.

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 Thermo Fisher. The 96-well black microplates with clear bottoms, catalogue number 3915, were purchased form Corning, (Corning, NY).

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 solution was prepared by first diluting concentrated buffer stock solution, provided in the NanoOrange® kit, 1:10 with distilled water according to the kit instructions[4]. This buffer was then subsequently used to dilute the NanoOrange® dye concentrate 1:1000 to make 1x working solution. After dilution of the protein samples, they were incubated for 10 minutes at 95 °C, and then allowed to cool to room temperature. Aliquots (200 µL) of the cooled samples were pipetted into microplate wells in replicates of eight. Fluorescence was determined using a BioTek Instruments Synergy Neo2 Multi-Mode Reader using either monochromators or filters cubes. The excitation wavelength with the monochromators was 485 nm with a 30 nm bandwidth, while emission was set to 590 nm, with a 30 nm bandwidth. Fluorescence filter cubes used 485/20 excitation and 590/30 emission filters in conjunction with a 510 nm cutoff dichroic mirror.

BioTek Instruments, Inc.
P.O. Box 998, Highland Park,
Winooski, Vermont 05404-0998 USA
Phone: 888-451-5171
Outside the USA: 802-655-4740
Email: **customercare@biotek.com**
www.biotek.com

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 Gen5™ data reduction software (BioTek Instruments), a 4-parameter non linear equation describing the curve can be generated. Lower concentrations demonstrate a linear response. When the linearity of the reaction is examined, a least means squared straight line can be utilized for DNA concentrations up to 1000 ng/mL with very high confidence.

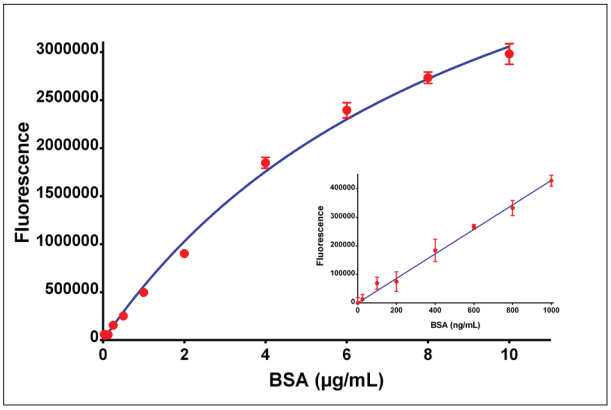


Figure 1. **Linearity of Filter-based Detection.** Concentration curve from 0.0 to 10 µg/mL of BSA with 4-parameter regression analysis. Insert figure depicts the data points for the lower protein concentrations (0 to 1000 ng/mL) with a linear regression analysis. Data points represent the mean values 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 lowest protein concentrations tested (data not shown). Although the curve is non-linear, determinations can be made with a high level of confidence (r^2 = 0.995) using a 4-parameter logistic fit of the data.. In terms of sensitivity, the reaction was found to be sensitive to the nanogram level, with fluorescent intensity values for 25 ng/mL being statistically different from the blank (P<0.002). The coefficient of determination (r^2=0.997) of the linear regression indicates that concentration determinations can be made with a high degree of confidence at these very low concentrations.

In addition to filter cubes for fluorescence detection, the Synergy™ Neo2 can be configured with dual monochromators for both excitation and emission. Similar linearity can be observed using the monochromators for wavelength selection as compared to the filter-based detection (Figure 2).

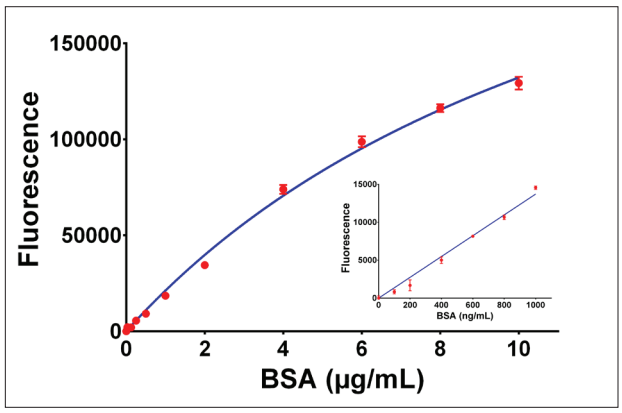


Figure 2. **Linearity of Monochromator-based Detection.** Concentration curve from 0.0 to 10 µg/mL of BSA with 4-parameter regression analysis. Insert figure depicts the data points for the lower concentrations (0 to 1000 ng/mL) with a linear regression analysis. Data points represent the mean values of eight determinations at each concentration.

Despite the lower magnitude in raw scale, the monochromators provide a better signal to background ratio as compared to the filters, as demonstrated in Figure 3. This is primarily the result of lower background measurements produced by the dual monochromators. With either optical system a 20-fold or greater assay window is observed.

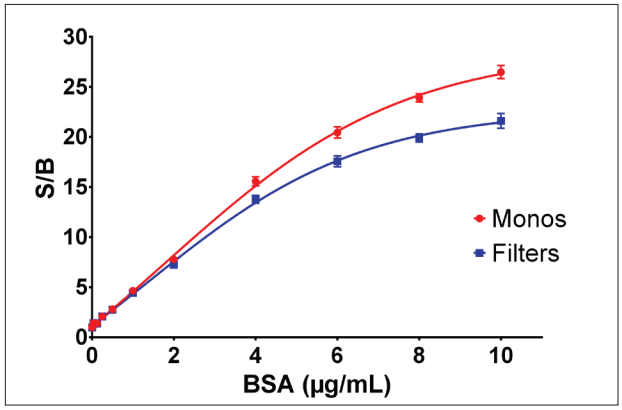


Figure 3. **Signal/Background Comparison of Monochromator- and Filter-based Detection.** The ratio of each sample to the mean of the blank (0 µg/mL BSA) was plotted. Data represents the mean and standard deviation of eight determinations.

This fluorescent total protein assay in conjunction with BioTek's Synergy™ Neo2 Multi-Mode Reader offers several advantages. Because the SDS detergent in the working solution maintains the denatured condition of the protein following heating, samples can be read hours later with no loss of sensitivity if protected from light. Like most assays that are read in microplates, the ability to read all of the samples rapidly 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 cost per assay by reducing the amount of expensive reagents necessary.

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 Thermo Fisher, Inc https://tools.thermofisher.com/content/sfs/manuals/mp06666.pdf