Application Note

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# Protein Quantitation Using NanoOrange Fluorescence

## Using the Agilent BioTek Synergy Neo2 hybrid multimode reader to quantify total protein

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## 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. This application note describes a fluorescent method to quantitate total protein using Agilent BioTek's Synergy Neo2 hybrid multimode 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 specific 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. This application note describes a fluorescence method to quantify total protein in the 96-well microplate-based format using the Synergy Neo2 hybrid multimode 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, catalog number 20279 from Thermo Fisher Scientific (Waltham, MA), 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.

## Experimental

A NanoOrange protein quantitation kit, catalog number N-6666, was purchased from Thermo Fisher Scientific (Waltham, MA). The 96-well black microplates with clear bottoms, catalog number 3915, were purchased from Corning Inc. (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. The 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 1:1,000 to make 1x working solution. After dilution of the protein samples, they were incubated for 10 minutes at 95 ºC 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 the Synergy Neo2 hybrid multimode 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.

## Results and discussion

The fluorescence intensity was determined for BSA protein concentrations ranging from 0.0 to 10 µg/mL, as seen in Figure 1. Over this range, the fluorescent intensity increased in a hyperbolic fashion. Using the Agilent BioTek Gen5 microplate reader and imager software, a 4-parameter nonlinear 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 used for DNA concentrations up to 1,000 ng/mL with very high confidence.

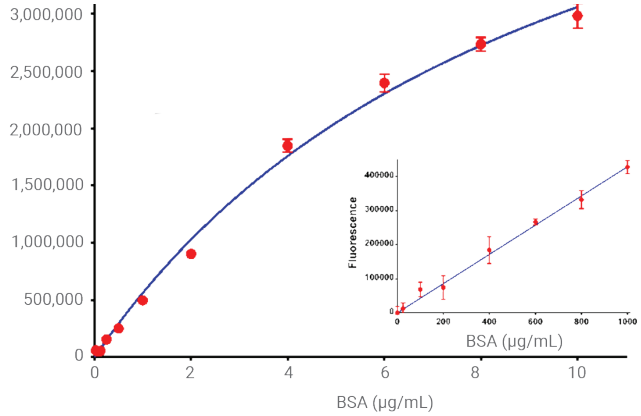


**Figure 1.** Linearity of filter-based detection. Concentration curve from 0.0 to 10 µg/mL of BSA with 4-parameter regression analysis. The inserted figure depicts the data points for the lower protein concentrations (0 to 1,000 ng/mL) with a linear regression analysis. Data points represent the mean values of eight determinations at each concentration.

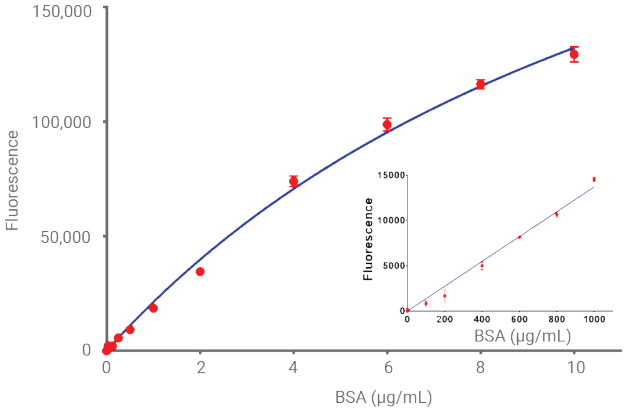


**Figure 2.** Linearity of monochromator-based detection. Concentration curve from 0.0 to 10 µg/mL of BSA with 4-parameter regression analysis. The inserted figure depicts the data points for the lower concentrations (0 to 1,000 ng/mL) with a linear regression analysis. Data points represent the mean 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, as shown in Figure 2.

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.

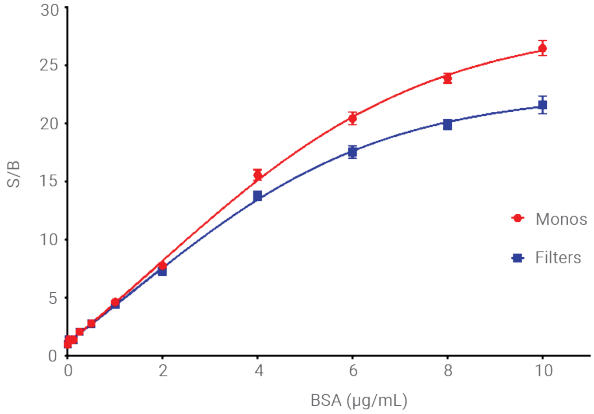


**Figure 3.** Signal/background comparison of monochromator- and filter-based detection. The ratio of each sample to the mean of the blank (0 µg/mL BSA) was plotted. Data represent the mean and standard deviation of eight determinations.

This fluorescent total protein assay, in conjunction with the Agilent BioTek Synergy Neo2 hybrid multimode 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


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