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Application Note

# Fluorescence-based Total Protein Determination using the Compound CBQCA

*Total protein content is a measurement common to many applications in basic science and clinical research. Several different fluorescent techniques are available that eliminate many of the problems associated with the traditional colorimetric absorbance-based methods to measure total protein content. Here we describe the use of 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) 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. For instance, the specific activity of an enzyme 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.

Over the years, many different absorbance-based colorimetric methods to quantify protein have been developed, the most utilized of which rely on the reduction of copper in the presence of a chromogenic reagent (1, 2). While these methods work well, they are subject to interference by many compounds commonly used in protein purification, namely detergents and reducing agents. Alternatively, simple absorbance measurements of protein solutions at 280 nm (A280) can be performed, but are subject to interference from any nucleic acid contamination. Dye-binding protein assays such as that described by Bradford (3) have also been developed. This assay is subject to the formation of aggregates leading to a loss of signal over time.
Several different fluorescent assays for protein quantitation have been developed in an attempt to alleviate the difficulties presented when using absorbance based assays. The compounds fluorescamine and o-phthaldialdehyde have been used with success to quantitate protein content of samples. Likewise, 3-(4-carboxybenzoyl) quinoline -2- carboxaldehyde (CBQCA) reacts with primary amines to form fluorescent moieties (Figure 1). This compound has the added advantage of functioning well in the presence of lipids, which normally interfere with protein determinations (5). Here we describe a fluorescent assay using CBQCA to quantitate total protein in the 96-well microplate based format using the BioTek FL600 fluorescence microplate reader in conjunction with KC4 data reduction software.

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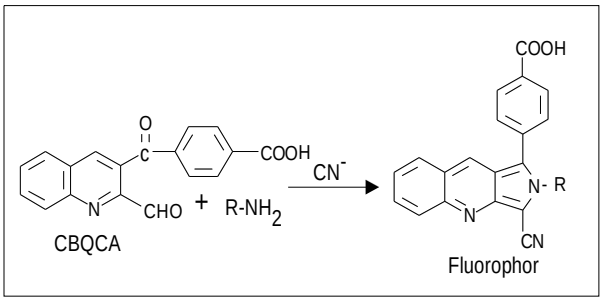


**Figure 1. Reaction of CBQCA with primary amines.** CBQCA, in the presence on CN- reacts with the terminal amino groups and the e amino groups of lysine to form fluorescent moieties.

## Materials and Methods

A CBQCA Protein Quantitation kit, catalogue number C-6667, was purchased from Molecular Probes (Eugene, OR). PBS powder packets (catalogue no. 1000-3) were obtained from Sigma Chemical Company (St Louis, MO) and the 96 well black microplates with clear bottoms (catalogue number 3603) were purchased from Costar (Cambridge, MA).

A series of dilutions ranging from 0.0 to 50 mg/ml of Bovine Serum Albumin (BSA) were made using phosphate buffered saline (PBS), pH 7.4, as the diluent. After dilution, 100 ml aliquots of samples and standards were pipetted into microplate wells in replicates of eight. After aliquoting the samples, 35 ml of PBS was added to each well followed by the addition of 5 ml of a 20 mM KCN solution and 10 ml of a 4 mM CBQCA solution bringing the final assay volume to 150 ml. The 4 mM CBQCA solution was prepared by dilution of a 40 mM CBQCA in DMSO stock solution with PBS immediately prior to use. Samples were protected from light by covering the plate with aluminum foil and were incubated at room temperature with shaking for 60 minutes. After incubation, the fluorescence was determined using a BioTek Instruments FL600 fluorescent plate reader with a 460 nm, 40 nm bandwidth, excitation filter and a 545 nm, 40 nm bandwidth emission filter. The sensitivity setting was at 42 and the data collected from the bottom with a 5 mm probe using static sampling with a 0.35 second delay, 50 reads per well.

## Results

As demonstrated in Figure 2, when reacted with CBQCA, protein concentration shows a direct correlation with fluorescence. The fluorescence intensity was determined for BSA protein concentrations ranging from 0.0 to 50 mg/ml. Over this range the fluorescence intensity increased in a linear fashion. Using KC4 data reduction software (BioTek Instruments), a 4-parameter logistic fit equation describing the standard curve was generated. The high correlation coefficient (r2=0.9998) indicates that determinations can be made with a high level of confidence over the entire concentration range tested. If a linear regression analysis is performed rather than a 4-parameter logistic fit, correlation remains exceptional with r2=0.995 (data not shown). This suggests that either data reduction algorithm can be used to determine protein concentrations of samples.

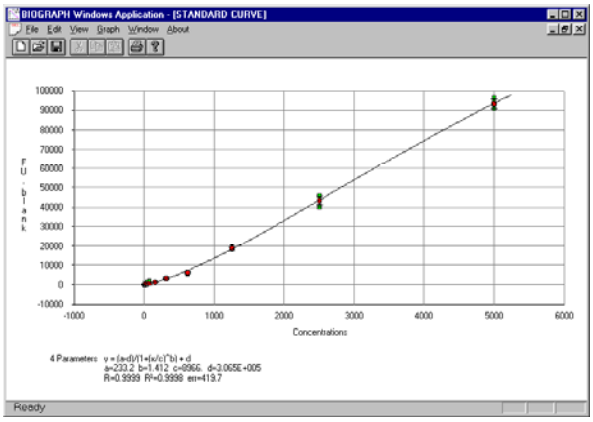


**Figure 2. Response of the assay.** Concentration curve from 0.0 to 5000 ng per well of BSA with 4-parameter logistic fit regression analysis. Data indicated has been corrected by subtraction of the 0 mg/ml blank.

## Discussion

The assay described in this report relies on the interaction of a compound and primary amines to form a fluorescent moiety. Primary amines exist in proteins at the amino terminus and at the e-position of the amino acid, lysine. Although all proteins contain an amino terminus, they have varying amounts of lysine residues depending on the protein and as such, different proteins would be expected to return different amounts of fluorescent signal from equivalent amounts of protein. Although it is optimal to use a calibration curve generated from the protein to be measured, this may not always be possible or warranted. For instance, in many cases the protein determination is performed on a mixture of many different polypeptides. However, in order to make comparisons between assays, it is important to be consistent with the protein used to make the calibration curve.

Several factors can influence the reactivity of CGQCA to proteins. The use of a buffer with a basic pH (pH 9.0 is optimal) results in greater fluorescence as primary amino groups are more likely to be protonated and thus more reactive. Fortunately, less than optimal pH levels including those around the physiologic range (pH 6-9) provide quite acceptable results. Many buffer systems in the pH range of 6-9, such as PBS or sodium borate, are suitable for this reaction; however, they should not contain amines (e.g., Tris or glycine). For these experiments PBS with a pH of 7.4 was used as a buffering agent.

The presence of thiol groups has been demonstrated to reduce the fluorescent signal (4, 5). Concentrations of dithiotheitol (DTT) above 1 mM interfere with the formation of the CBQCA-amine adduct (5). Similar results would be expected with the presence of b-mercaptoethanol in high concentrations. Although, low levels (less than 100 mM) of either reducing agent have been reported to not interfere with the reaction; if reducing agents are present in the unknown samples, it is advisable to use standards with equivalent amounts of reducing agent. Alternatively, the addition of N-ethylmaleimide (NEM), which reacts with free thiol, blocks thiol interference without itself interfering with protein determination (5). On the other hand, many other agents commonly added to protein extracts, including Triton X 100, cesium chloride, glycerol, SDS, and sodium azide do not seem to interfere with the assay (4, 5).

The compound CBQCA has been found to work exceptionally well with lipid-associated proteins. Lipoproteins (e.g. LDL,VLDL, and HDL) can be assayed without any manipulation to extract lipids or the addition of detergents (5). This reagent would be expected to work well for other samples, such as brain tissue extracts, where lipid levels are expectedly high.
The use of KC4 software to control the reader allows the user a great deal of flexibility in regards to data reduction capabilities. The software allows the user to define any configuration of plate

map necessary. With several different curve fit algorithms to choose from, regression analysis of the standards and the subsequent concentration determinations of samples can be accomplished with a high degree of confidence. Likewise, the software is capable of performing statistical analysis on sample groups, as well as any mathematical calculation required by the user.

**References**

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Rev. 2-20-01