Application Note

IR-based Protein Quantitation Surpasses Colorimetric Assay Quantitation and Results are Independent of Detergents, Reducing Agents and Analysis Time

Abstract

Accurate protein quantitation in complex mixtures is crucial for obtaining reproducible biological and biochemical data for both upstream and downstream applications. Colorimetric protein assays are frequently used to determine protein concentration; however, the calculated concentration can vary depending on the sample composition, sequence of the protein, and the time point within the chemical reaction at which the data are collected. Studies involving cell lysates and multiple, diverse proteins (such as multianalyte pathway elucidation) require the development of a rapid, universal protein quantitation method that is compatible with complex samples. Here, we compare infrared (IR)-based protein quantitation using the Direct Detect™ quantitation system to Bradford and bicinchoninic acid (BCA) colorimetric assays and show that IR-based quantitation provides results that match concentrations determined by amino acid analysis, even in the presence of detergent or reducing agent. We also show that, unlike colorimetric assays, the calculated protein concentration obtained from IR-based analysis is unchanged regardless of the time delay between assay and data acquisition.

Introduction

The most common colorimetric assays for protein quantitation involve protein-copper chelation (BCA and Lowry assays) and dye-binding based detection (Bradford and "660" assays). While these assays are easy to use, major disadvantages include the large variation in the binding efficiency to different proteins, reproducibility, and sensitivity to sample contaminants[1]. Intrinsic protein characteristics that can affect concentration estimates in colorimetric assays include amino acid content, post-translational modifications, and protein secondary and tertiary structure. In one study, colorimetric assays gave results up to 60% different from values derived from amino acid analysis[1].

Bradford assay and its limitations

The Bradford assay relies on binding of Coomassie Brilliant Blue G250 to protein[2]. This dye binds to basic amino acids, particularly arginine, and its absorbance shifts from 465 nm to ~595 nm upon binding. As a result, the assay response depends on the number of basic amino acid residues in the protein. Because the assay requires the generation of a standard curve for every experiment, the proportion of basic amino acid residues in the protein standard needs to be similar to that of the protein being assayed. Also, there may be a large variation in assay response between different preparations of the Bradford reagent. Specifically, it has been shown that the absorbance maximum of the dye-protein complex varies between 595 nm and 620 nm, depending on the dye source[3].

Furthermore, assay response is affected by detergents (such as those used to solubilize membrane proteins). Finally, the Bradford assay is nonlinear at the higher end of the recommended protein concentration range.

BCA assay and its limitations

The BCA assay involves a two-step chemical reaction. The first step requires protein binding to Cu^{2+}, which is reduced to Cu^{1+} by cysteine, tyrosine, and tryptophan residues, as well as by the peptide bond. In the second step, BCA chelates Cu^{1+} to form a purple complex that absorbs light at 565 nm. Because it is so sensitive to the amino acid composition of the protein, the BCA assay, like the Bradford assay, requires a standard curve for each experiment in which the protein standard has comparable amino acid composition to the protein being measured. For maximum sensitivity, the BCA assay requires heating, and assay signal can change depending on the length of incubation. The BCA assay is compatible with samples containing up to 5% ionic detergents; however, phospholipids[4], chelating agents, reducing agents, and certain nonionic, oxidizing detergents can affect the assay signal.

Amino acid analysis (AAA)

Considered one of the most accurate methods of protein quantitation, AAA involves hydrolysis of protein into individual amino acids. Amino acids are then derivatized with ninhydrin, a chromogenic molecule, and chromatographically separated. The ninhydrin-labeled amino acids are then detected at 440 and 570 nm. Protein concentration is calculated based on the signal generated using a standard of precisely known amino acid composition and concentration.

IR-based quantitation

IR spectroscopy exploits the fact that molecules absorb radiation at specific frequencies characteristic of their structure and functional groups. Protein primary structure (common to all proteins) is formed by a long chain of amide (peptide) bonds. Amide bonds absorb radiation in multiple regions of the IR spectrum, including the amide I band at 1600–1690 cm^{-1}. In order to determine protein concentration, the Direct Detect™ system calculates the intensity of the amide I band. Amide bond quantitation is not subject to interference from many common buffer components, such as detergents, reducing agents and chelators. Therefore, we hypothesized that the Direct Detect™ system could be used to measure protein concentration with accuracy and precision in complex samples and over extended time periods.

Materials and Methods

The concentration of a stock solution of bovine serum albumin (BSA, Cat. No. 126609) was verified by submitting samples for amino acid analysis. This solution was then diluted to 2 mg/mL with phosphate-buffered saline (EmbryoMax® PBS, Cat. No. BSS-1006-A), 50 mM dithiothreitol (DTT, Life Technologies, Cat. No. NP0004), and 1% SDS (Hoefer, Cat. No. GR155-1). This solution was then serially diluted to obtain 1.5, 1.0, and 0.5 mg/mL samples. The concentrations of the resulting 4 BSA samples were measured using BCA assay, Bradford assay, and IR-based quantitation.

Bradford and BCA assays were performed in 96-well Corning Costar® UV plates using the Coomassie Plus Protein Assay Reagent (Thermo Scientific, Cat. No. 23238) and Pierce Micro BCA™ Protein Assay (Thermo Scientific, Cat. No. 23235), respectively. For each assay, a standard curve was generated using prediluted BSA standards (Thermo Scientific, Cat. No. 23208). Colorimetric measurements were made using a Biotek® UV plate reader.

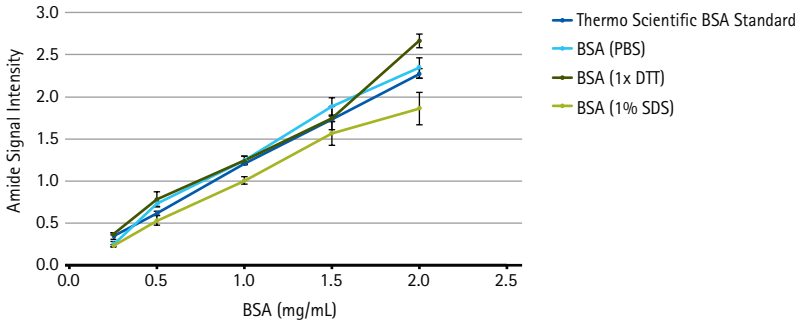
IR-based protein quantitation was performed using the Direct Detect™ spectrometer (Cat. No. DDHW00010-WW) which was calibrated once using the same prediluted BSA standards. For each protein sample, 2 µL was spotted on a Direct Detect™ Assay-free Card (Catalogue No. DDAC00010-8P). The card was inserted into the instrument and results recorded using the included software.

To assess the effects of time on observed protein concentration measurements, Micro BCA™ and Coomassie Plus Protein Assay plates were read at time "0" (the time at which the incubation period was complete) and time "30 min" (30 minutes after incubation step/time 0 read). The assay signals from the prediluted BSA standards were plotted at each time point. To assess the effect of time on IR-based protein quantitation, Direct Detect™ assay-free cards spotted with the prediluted BSA standards were re-analyzed approximately 60 hours after the initial data acquisition.

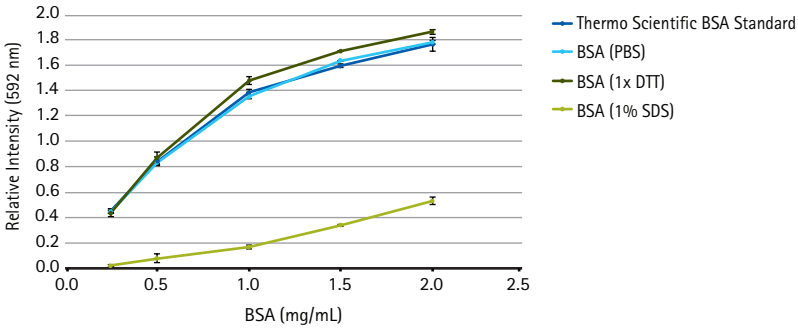
Results

As shown in Figure 1, only IR-based quantitation yielded calculated concentrations for the AAA-verified BSA samples that correlated with the prediluted standards for all sample compositions tested (PBS, PBS + 50 mM DTT, and PBS + 1% SDS). The Coomassie Plus (Bradford) assay provided inaccurate quantitation in the presence of 1% SDS, and the Micro BCA™ assay could not provide quantitation in the presence of 50 mM DTT.

1A. Direct Detect™ IR-based Quantitation



1B. Coomassie Plus Assay



1C. Micro BCA™ Assay

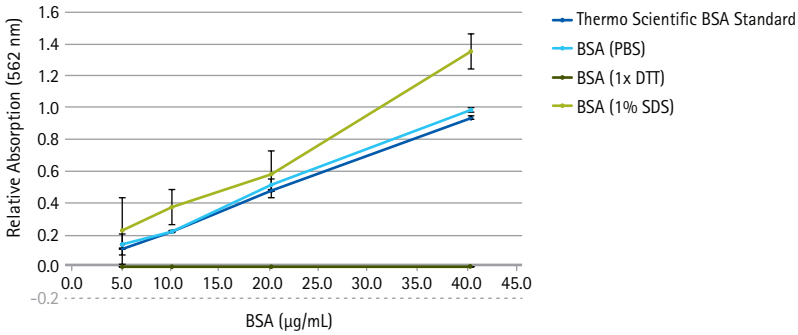
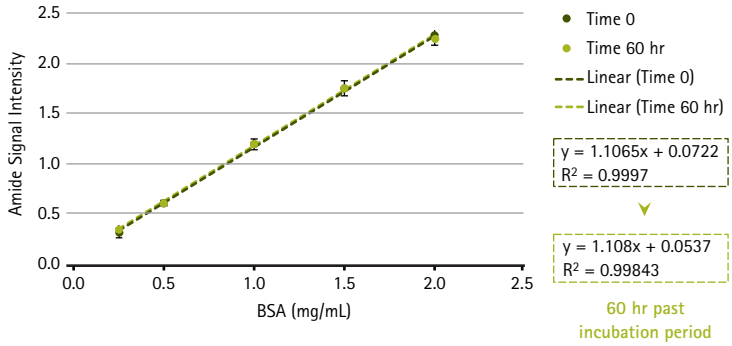


Figure 1. IR-based quantitation (A) provides accurate and precise results, even in presence of detergent (SDS) and reducing agent (DTT). Using the Direct Detect™ IR-based quantitation system, calculated concentrations for the BSA samples matched the prediluted standards (A). In comparison, the Coomassie Plus (Bradford) assay provided calculated concentrations that differed greatly in the presence of 1% SDS (B), and the Micro BCA™ assay could not provide data in the presence of 50 mM DTT. (C).

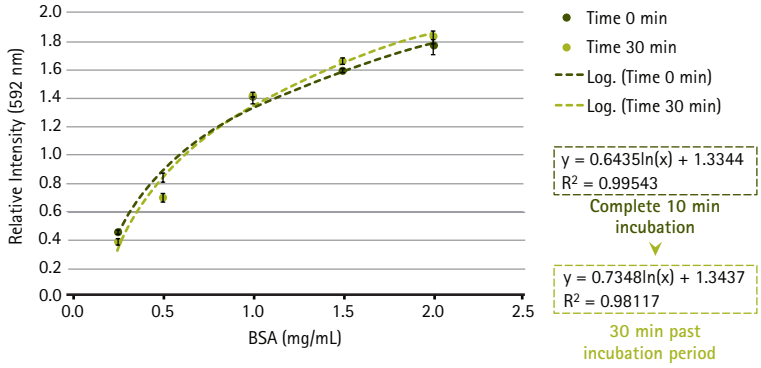
Next, we examined the effect of time on protein quantitation using the three methods in Figure 2. IR-based quantitation using the Direct Detect™ system yielded calculated protein concentrations for the prediluted BSA standards that were the same at both time 0 and 60 hours later. The linear fitted standard curves for both time points were superimposable, without statistically significant differences (Figure 2A). In contrast, for the Coomassie Plus (Bradford) assay, when the standards were measured 30 minutes after the initial measurement, the resulting fitted curve equation differed from the original equation (Figure 2B). Similarly, for the Micro BCA™ assay, the slope of the fitted curve increased by 16% upon assay incubation for an additional 30 minutes (Figure 2C).

Figure 2. IR-based protein quantitation is less time-dependent than colorimetric assays. Prediluted BSA samples were quantitated at the indicated times using IR-based quantitation (A), Coomassie Plus (Bradford) assay (B), and Micro BCA™ assay (C). Protein concentrations calculated using IR-based quantitation remain unchanged even after spotted samples are left in ambient conditions for 60 hours, while calculated concentrations from the kinetically dependent colorimetric assays continue to change over time.

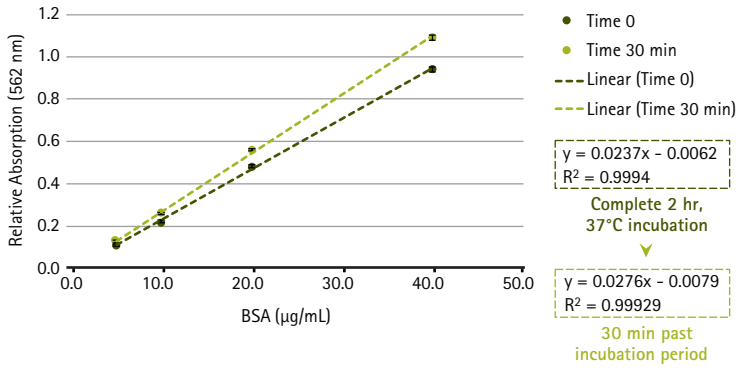
2A. Direct Detect™ IR-based Quantitation



2B. Coomassie Plus Assay



2C. Micro BCA™ Assay



Discussion

IR-based protein quantitation using the Direct Detect™ system involves measuring the intensity of the Amide I peak in the protein's IR spectrum, and subtracting the signal contributed by buffer alone in that region. A sample spectrum (Figure 3) shows that the IR spectrum of SDS does not have a strong signal in the Amide I region that would significantly interfere with protein quantitation. As a result, IR-based quantitation retains accuracy and reproducibility in the presence of SDS. Similarly, the IR spectra of reducing agents such as DTT do not interfere with Amide I quantitation (spectra not shown).

We have also shown that IR-based quantitation of proteins is independent of time. Unlike colorimetric Bradford and Micro BCA™ assays, which are based on indirect detection of a secondary reaction and whose signals continue to change over time, the IR signal of a protein does not depend on reaction kinetics.

The stability of the IR signal over time, together with compatibility with detergents and reducing agents, make IR-based quantitation a more convenient, flexible, universal approach to measuring protein levels in complex mixtures, compared to the Bradford and BCA colorimetric assays. Also because assay-free, IR-based quantitation only requires a standard curve to be generated once (instead of for every experiment), this method is also faster than quantitation using colorimetric assays. Benchmarking every experiment to the same, robust standard curve also provides more reproducible results and facilitates intra-assay comparisons across multiple experiments referencing the same standard curve.

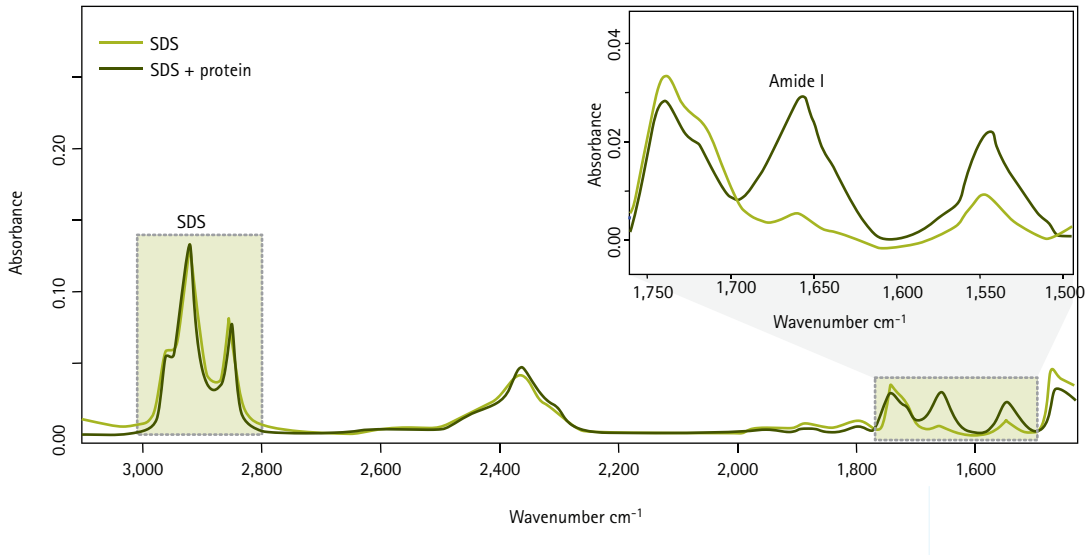


Figure 3. The characteristic peaks of the SDS IR spectrum are distinct from the Amide I region of the protein spectrum.

References

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4. Kessler, R.J., Fanestil, D.D. (1986). Interference by Lipids in the Determination of Protein Using Bicinchoninic Acid. Analytical Biochemistry, 159:138-142.

Ordering Information

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