Application Brief

Molecular Biology and Biochemical

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Protein Analysis Using Microplate-Based Quantification Methods

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Introduction

The use of spectrophotometers has long been a staple in scientific laboratories for the analysis of a wide variety of sample types. In many applications it is possible to take advantage of the physical properties of an analyte in solution to quantify the amount present. Methods that take advantage of these properties in a sample in its native state may be nondestructive and are preferable, allowing recovery of the sample for use in downstream applications. Proteins are one such example of a biomolecule with an inherent ability to absorb light that directly correlates to the amount present. However, in some instances sample availability is limited, resulting in samples of very low concentration that require a reporter molecule that is directly proportional to the analyte present, therefore extending the quantifiable concentration range. Several reagents are available, allowing quantification of such samples including both the Bicinchoninic Acid (BCA) and Bradford assays. Both reagents rely on absorption of light of a specific wavelength based on a colorimetric reaction that is easily detected by a spectrophotometer such as the Agilent BioTek Synergy LX multimode reader.

Methods

UV absorption

Purified bovine serum albumin (BSA) was used to prepare a stock solution of ~10 mg/mL in MilliQ water. A serial dilution was then prepared from the stock ranging from 0 to 1,500 µg/mL. Each dilution was then analyzed in a 96-well, UV-transparent microplate in a 100 µL volume. The Beer-Lambert Law relates the absorption and concentration via the following equation where A = log Io/I = εlc. ε refers to the extinction coefficient of the analyte, l is the path length (cm) and c is the concentration of the analyte (ng/µL). The Agilent BioTek Gen5 microplate reader and imager software has built-in methods for path length correction to 1 cm for samples diluted in aqueous buffers in variable path lengths vessels. The Agilent BioTek Synergy LX multimode reader with absorbance monochromators was used to measure absorbance at 280, 320, 900, and 977 nm in a single protocol to allow automated path length correction and protein quantification.

BCA assay

Bicinchoninic Acid Kit and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO). Protein standards were prepared as described above covering a suitable concentration range for the assay. The BCA assay was performed in a 96-well microplate as per the manufacturers' recommendations. Briefly, 25 µL of protein standard or sample is added to the microwell followed by the addition of 200 µL BCA working reagent (1:8). The microplate is sealed and allowed to incubate at 37 °C for 30 minutes. Absorbance was determined at 562 nm using an Agilent BioTek Synergy LX multimode reader.

Bradford assay

Bradford Assay Reagent and BSA were obtained from Sigma-Aldrich and standards prepared as described above covering a suitable concentration range for the assay. Briefly, 5 µL of protein standard or sample is added to the microwell followed by the addition of 250 µL of Coomassie reagent. The microplate is sealed and allowed to incubate at room temperature for 10 minutes. Absorbance was determined at 595 nm using an Agilent BioTek Synergy LX multimode reader.

Results and discussion

The use of native and colorimetric absorbance methods provides a means to quantify proteins over a wide range of concentrations. UV absorbance is commonly used being considered an easy, nondestructive method for quantification of proteins (Figure 1). However, the limit of detection can preclude analysis of samples from many sources given the low yields and subsequent very low concentrations typically encountered. Additionally, many interfering compounds and biomolecules may be present from sources such as elution buffers that will skew the resultant determinations.

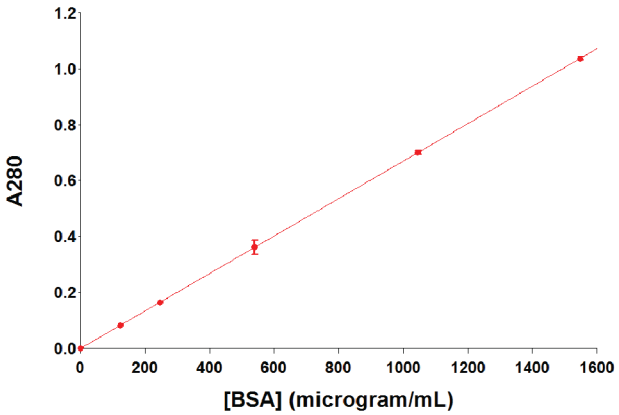

Figure 1. A_{280} measurements. A dilution series of BSA was prepared ranging from 0 to ~1,500 µg/mL in MilliQ water. Standards were analyzed in triplicate.

More sensitive assay technologies using colorimetric reagents that are relatively unaffected by contaminants can be used to accurately quantify protein concentrations. The BCA assay is one such method based on the biuret reaction where BCA reagents act to form a purple-colored product attributed to the chelation of two molecules of BCA per Cu^{+} ion. The assay signal is proportional to the number of exposed peptide bonds in a protein and is less affected by protein structural variants and contaminating biomolecules in comparison to UV methods. The BCA assay is compatible with many surfactants typically used for protein stabilization. The use of protein standards of the target protein for generation of standard curves allows for the determination of sample concentrations (Figure 2). The BCA assay in the 96-well microplate format has a calculated limit of detection of ~157 µg/mL for BSA.

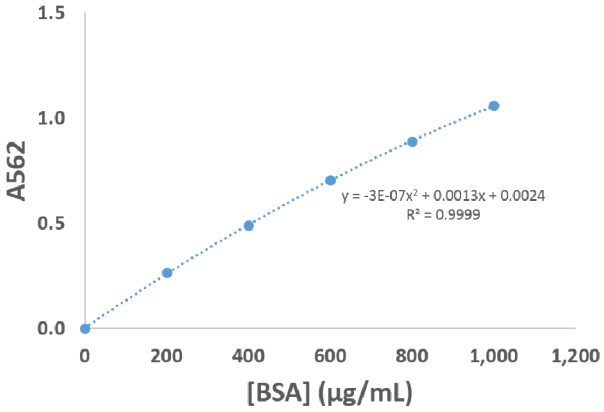

Figure 2. BCA assay. A BCA protein standard curve was produced using a dilution series of BSA.

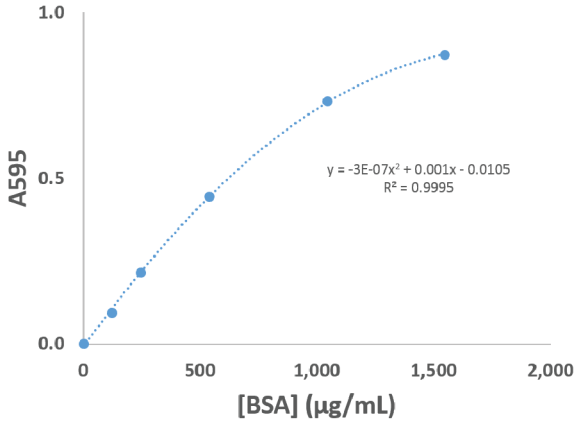

Figure 3. Bradford assay. A Bradford protein standard curve was produced using a dilution series of BSA.

In a similar fashion, the Bradford assay relies on a colorimetric readout to extend the dynamic range of protein detection. The Bradford assay generates color development at 595 nm due to binding of Coomassie Blue G-250 to peptides in an acidic medium and is faster and more stable than the BCA assay. However, the response will vary with protein primary structure as the reaction relies on ionic bonding with nearby positively charged amine groups. Additionally, elevated detergent levels and buffer conditions may interfere with color formation and possibly result in precipitate formation. In the same manner as in the BCA assay, the use of protein standards of the target protein for generation of standard curves allows for the determination of sample concentrations (Figure 3). The Bradford assay in the 96-well microplate format has a calculated limit of detection of ~138 µg/mL for BSA. The assay can also be performed in a low range format further extending the dynamic range to submicrogram per mL concentration determinations (data not shown).

Conclusion

The Agilent BioTek Synergy LX multimode reader provides the most common detection technologies used in biological research including absorbance, fluorescence, and luminescence detection. This application brief demonstrates its utility to quantify proteins using UV detection and various colorimetric assays to extend the dynamic range of measurable concentrations and possessing tolerance to many interfering compounds.

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