Application Note

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Analytical Performance of Agilent BioTek Epoch Multivolume Spectrophotometer System for Protein Quantification

Authors

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Abstract

The accurate determination of protein concentration is necessary for a large number of downstream applications. The Agilent BioTek Epoch multivolume spectrophotometer system allows the use of a variety of such methods including native protein absorbance at 280 nm and colorimetric reagents such as bicinchoninic acid to discern a large dynamic range of concentrations over a wide range of sample volumes on a single instrumentation platform.

Introduction

The purification and accurate quantification of proteins remain central to diverse applications ranging from protein expression profiling using LC/MS/MS, two-dimensional (2D) gel electrophoresis, and recombinant protein expression for reagent tool development. Proteins are typically quantified either by absorption at 280 nm or with the aid of signal enhancement reagents such as bicinchoninic acid (BCA).[1] BCA reagents act in conjunction with the biuret reaction to form a purple colored product attributed to the chelation of two molecules of BCA per Cu^{+} ion. Strong absorption of 562 nm light provides for an assay that is typically more sensitive than native protein absorbance at 280 nm. BCA assays also tend to be less prone to interference from other UV absorbing material, such as nucleic acids. Conversely, native protein absorbance at 280 nm is a simple assay and tends to provide a more linear response to protein concentration ranges.

Recently, microvolume absorbance-based protein quantification using extremely short measurement pathlengths, has been demonstrated with dedicated instruments such as NanoDrop (Thermo Fisher Scientific). This instrument and methods inherent are particularly useful using native protein absorbance at 280 nm due to sample conservation and ease-of-use.

This application note shows the utility of the Epoch multivolume spectrophotometer system (Figure 1) for microvolume protein analysis using either native protein absorbance at 280 nm or with the use of BCA. Microvolume results were also compared to those from NanoDrop 2000c (Thermo Fisher Scientific).

Figure 1. Agilent BioTek Epoch multivolume multisample spectrophotomer system provides a versatile platform for quantifying protein samples covering a wide dynamic range of concentrations and detection methods. The Agilent BioTek Take3 multivolume plate allows measurement using a wide range of sample or assay volumes.

Material and methods

Native protein absorbance at 280 nm

All protein standards were created by preparing a 9 point 1:2 serial dilution series of a concentrated stock of bovine serum albumin (BSA) (part number A3294) from Sigma-Aldrich (St. Louis, MO) in Milli-Q water from MilliporeSigma (Burlington, MA). Epoch multivolume spectrophotometer system microvolume data were obtained with undiluted standard samples using the Take3 plate.

Each standard was loaded 5 times at each microspot location on the Take3 plate using an 8-channel manual pipettor. Optical densities were measured at 280, 260, and 320 nm resulting in 80 replicate determinations at each location. Agilent BioTek BioCell data was acquired either using undiluted standard samples for the lower concentration range or after 1:20 dilution in Milli-Q water for higher concentrations. NanoDrop microvolume data was determined from 10 replicate measurements of each of the same protein standards. All measurements were background subtracted using a water blank. All concentrations are based on a 1 cm pathlength and 0.667 mg/mL/OD extinction coefficient.

The limit of detection is defined as the analyte concentration providing a signal three-fold higher than the noise (standard deviation) of the background signal. The standard deviation for each microspot of the Take3 plate was determined from 10 measurements of reloaded blank solution. The 280 nm signals were corrected bichromatically at 320 nm as indicated above.

BCA colorimetric assays

The BCA assay was run in a format recommended by the NanoDrop technical manual. Briefly, the BCA assay was made by mixing 80 µL BCA working reagent with 4 µL protein standards and samples (20:1 ratio) in microtubes, incubations were performed at 37 °C for 30 minutes, then 2 µL loaded into either the Agilent BioTek Take3 plate in duplicate or on the NanoDrop pedestal as single measurements. For all assays, the absorbance of each standard and sample was read at 562 nm within 10 minutes of each other. All measurements were blank corrected.

Bovine serum albumin (BSA) protein standards were prepared as a 7 point 1:2 serial dilution series resulting in concentration in the range from ~0.100 to 2 mg/mL. Absorbance measurements were taken using the Agilent BioTek Take3 and Thermo Fisher Scientific NanoDrop in a standard UV-transparent quartz cuvette and compared to measurements taken in a BioCell on Take3.

Results and discussion

Native protein absorbance at 280 nm

Linear dynamic range

The linear dynamic range of the Agilent BioTek Epoch multivolume spectrophotometer system for microvolume analysis spans more than three orders of magnitude covering a concentration range from ~0.2 to ~50 mg/mL as shown in Figure 2. This linear dynamic range is consistent with NanoDrop.

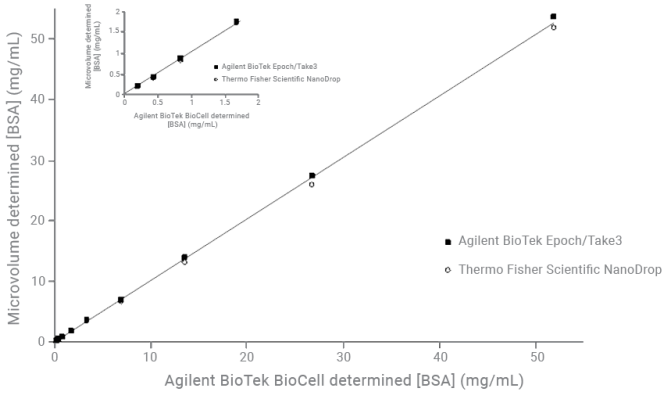

Figure 2. Protein standard curve using dilutions of bovine serum albumin measured at 280 nm. Abscissa data is considered actual [protein] as measurements were performed with 1 cm pathlength in BioCell. Ordinate data are microvolume determinations using either Agilent BioTek Epoch multivolume spectrophotometer system and Agilent BioTek Take3 plate or NanoDrop. The inset is an exploded view of low protein concentration. In both graphs, the line fit represents a slope of 1.000 and demonstrates equivalence between microvolume and 1 cm pathlength determinations.

Precision and accuracy

Protein concentrations derived from purification kits can vary considerably depending on starting material, purification method, and elution volume. Seeing sample concentrations ranging over three orders of magnitude is not uncommon. The precision of measurements taken over such a large dynamic range may also differ. With reduced precision, often comes reduced accuracy unless a large number of replicate measurements are used.

Figure 3 portrays the precision in the replicate measurements made to create the standard curves in Figure 2 and the accuracy relative to the 1 cm pathlength determination made with BioCell.

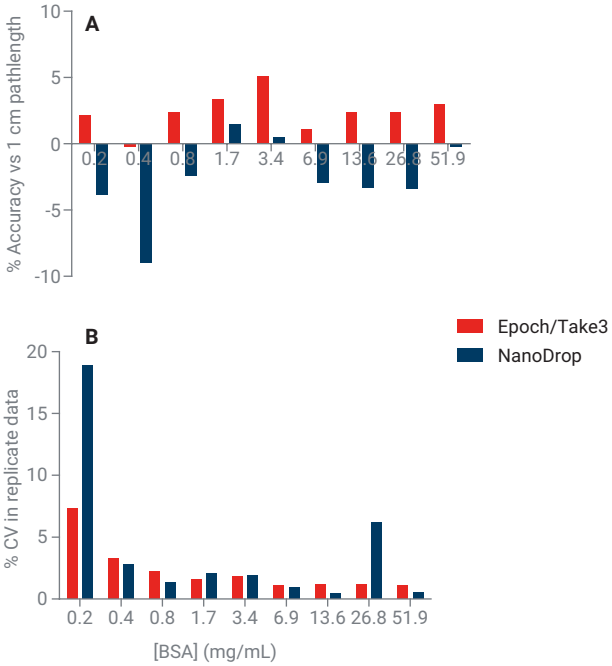

Figure 3. Comparison of microvolume methods for accuracy relative to 1 cm pathlength determinations of BSA concentration using BioCell (A) and precision in the replicate measurements made at each BSA standard (B).

Accuracy relative to 1 cm pathlength determinations is approximately the same for both microvolume instruments, although there appears to be greater accuracy at lower BSA concentrations using an Agilent BioTek Epoch microplate spectrophotomer. In general, however, Agilent BioTek Epoch/Take3 tends to provide slightly higher concentrations and NanoDrop slightly lower concentrations relative to BioCell. Each microvolume instrument possesses precision ≤2% CV ranging from high BSA concentrations down to approximately 0.5 mg/mL, although some of the high BSA concentration data from NanoDrop are subject to poor precision. Both instruments begin to lose precision <0.5 mg/mL, but NanoDrop data is significantly poorer at the lowest BSA concentration.

Limit of detection

Quantification of limits of detection has been discussed previously for the Agilent BioTek Take3 plate using nucleic acid samples.[2] The detection limit has been calculated to be 0.0021 OD, representative of three times the average standard deviation of the blank from the 16 microspots. The detection limit expressed as a protein concentration can be determined by using the data generated for the linear dynamic range. The lowest concentration of BSA used was determined to be 0.214 mg/mL using the BioCell. The average background corrected absorbance signal generated by the 16 Take3 microspots was determined to be 0.078 OD. Therefore, the detection limit for BSA is 6 µg/mL.

BCA colorimetric assay

Improved sensitivity relative to A280 measurements

Figure 4 demonstrates the improved analytical sensitivity obtained by using the BCA reagent relative to native protein absorbance at 280 nm.

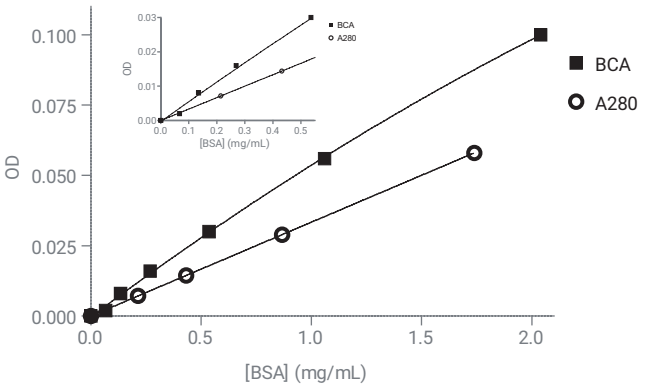

Figure 4. Comparison of microvolume standard curves using BCA (OD measurements at 562 nm) and A280 native protein absorbance using Agilent BioTek Epoch/Take3. The BCA calibration curve was fit with a quadratic equation as specified by the kit manufacturer; the A280 calibration curve used linear regression. Inset is an exploded view of the lower BSA concentration range. OD values are representative of a nominal pathlength of 0.5 mm.

The BCA standard curve is best fit with a quadratic equation; while the A280 data are linear, with a correlation coefficient of 1.0000. The greater slope of the calibration curve and a relative freedom from contributing signals from impurities such as nucleic acids, tends to provide BCA analysis with a greater accuracy for protein quantification relative to A280 measurements using real samples (i.e. cell lysates). The A280 analysis is a simpler workflow that does not require the purchase and addition of colorimetric reagents.

Comparison to NanoDrop

Figure 4 shows a comparison of microvolume BCA standard curves using both Agilent BioTek Epoch/Take3 and NanoDrop using the same standards and workflows.

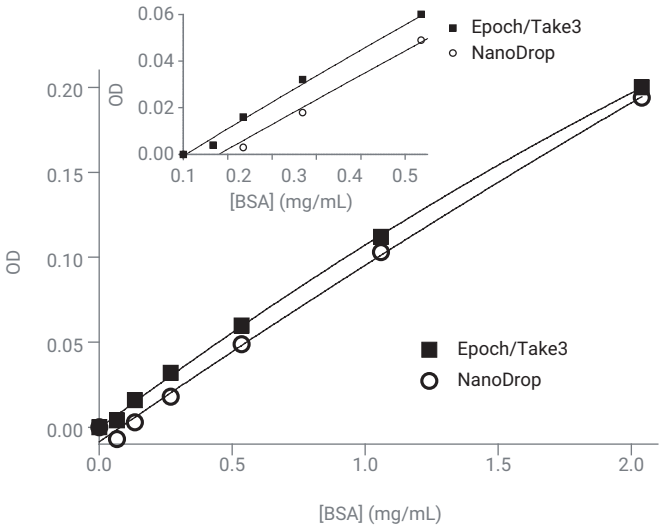

Figure 5. Comparison of microvolume BCA standard curves using Agilent BioTek Epoch/Take3 and NanoDrop 2000c. Each BCA calibration curve was fit with a quadratic equation. Inset is an exploded view of the lower BSA concentration range. OD values are representative of a nominal pathlength of 1 mm, consistent with NanoDrop software manipulations.

Both instruments possess a similar sensitivity as determined from the slope of the calibration curve, although the Agilent BioTek Epoch/Take3 appears to have an extended range in the low protein concentration range in this experiment.

NanoDrop software provides integrated assay protocols for a number of colorimetric reagents, including BCA. Collecting replicate data for each protein concentration is not possible, therefore comparisons in precision were not made in this application note. Rather, accuracy measurements were made at two protein standards, one at low BSA concentration where A280 measurements tend to suffer poor precision; the other at high BSA concentration. Table 1 depicts the comparisons between the two microvolume instruments.

Table 1. Comparison of accuracy in BSA determinations using BCA colorimetric reagent.

Instrument	[BSA] (mg/mL)*	A562	Calc. [BSA] (mg/mL)	% Accuracy
Agilent BioTek Epoch/Take3	0.269	0.023	0.254	-5.6
	1.06	0.093	1.013	-4.4
NanoDrop	0.269	0.021	0.298	10.8
	1.06	0.105	1.133	6.9

*BSA determinations made with a 1 cm pathlength and used as a reference point in the accuracy determinations.

The Agilent BioTek Epoch/Take3 has improved accuracy relative to NanoDrop in this experiment, especially at low protein concentration.

Conclusion

This application note shows the ability to use native absorbance and colorimetric methods to accurately determine protein concentration using the Agilent BioTek Epoch multivolume spectrophotometer system. When using native protein absorbance at 280 nm to quantify protein, the Agilent BioTek Epoch multivolume spectrophotometer system provided accurate microvolume analysis across a broad range of concentrations spanning three orders of magnitude. Precision and accuracy compared to 1 cm pathlength determinations over this range compared favorably to the NanoDrop. A detection limit of 6 µg/mL was observed when measuring purified protein in Milli-Q water.

The use of BCA reagent also provided the ability for Agilent BioTek Epoch/Take3 to characterize protein concentrations across three orders of magnitude with significantly greater sensitivity relative to native protein measurements using A280. Comparisons to NanoDrop were again favorable, especially at lower protein concentrations.

References

1. Smith, P.K., *et al.* Measurement of Protein Using Bicinchoninic Acid. *Anal. Biochem.* **1985**, *150*, 76–85.
2. Brescia, P.; Banks, P. Analytical Performance of Nucleic Acid Microvolume Quantification using the Epoch Spectrophotometer System, *Agilent Technologies application note*, Dec. **2009**.

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