

Abstract

Accurate determination of molecular concentrations is a prerequisite to the use of purified biomolecules for a multitude of downstream applications. Quantification is routinely accomplished by spectrophotometric analysis. BioTek recently introduced an accessory for its microplate spectrophotometers that can accommodate a range of liquid volumes and samples for spectrophotometric analysis. The new Take3™ Multi-Volume Plate incorporates the ability to measure up to sixteen low-volume (2 µL) samples for direct quantification, two vertical 1 cm pathlength cells and one standard 1 cm cuvette, in a standard microplate sized format. Its use allows a researcher to quantify samples without dilution while also allowing more dilute samples to be read in a standard 1 cm pathlength format.

Spectrophotometric measurements are derived from the low volume microspots, minimum 2 µL, a 1 cm pathlength vertical quartz cuvette and a 1 cm pathlength cuvette on board the accessory. The analytical performance of the accessory will be discussed to include limit of detection, dynamic range and accuracy for proteins and nucleic acids.

Introduction

Applications such as protein profiling using LC-MS/MS or RT-PCR, sequencing and micro-array analysis of nucleic acids rely heavily on accurately determined molecular concentrations of purified biomolecules. Current biomolecular isolation protocols and kits result in yields ranging from nanogram to milligram amounts and are typically eluted in 10-100 µL volumes. Therefore, typical concentrations can range from sub ng/µL to thousands of ng/µL.

Quantification is routinely accomplished by spectrophotometric analysis at 280 nm for proteins and at 260 nm for nucleic acids in a UV transparent vessel. Measurements are traditionally made in quartz cuvettes with a fixed pathlength of 1 cm and are typically associated with high precision and accurate measurements. Using standard 1 cm pathlength cuvettes, however, often requires diluting nucleic acid samples above about 100-200 ng/µL and protein samples above about 4 mg/mL. More recently, microplates have been used to determine analyte concentrations in a more condensed format of 96 samples per plate with a variable pathlength dependent on well diameter and sample volume.

Here we describe the use of a multi-volume plate for rapid measurement of multiple undiluted samples with volumes as low as 2 µL, each with 0.5 mm nominal pathlength. The multi-volume plate also provides the capability to make measurements using a standard 1 cm pathlength cuvette and/or two vertical 1 cm pathlength cuvettes (Figure 1).

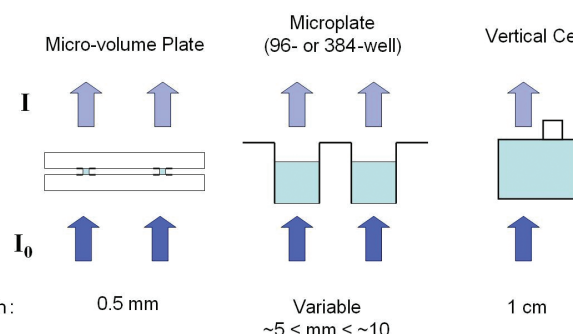


Figure 1 – Multi-volume analysis. Diagrammatical representation of various vessels and associated pathlengths used for spectroscopic measurement of biomolecular samples.

We show the analytical performance of the multi-volume plate for micro-volume protein analysis using either native protein absorbance at 280 nm or with the aid of the signal enhancing reagent bicinchoninic acid (BCA). Additionally, we illustrate the analytical performance of the accessory for micro-volume analysis using UV absorption for both dsDNA and RNA. The analysis includes determination of linear dynamic range, precision and accuracy, and limit of detection.

Material & Methods

Native Protein Absorbance at 280 nm

Linear Dynamic Range

All protein standards were created by preparing a 9 point 1:2 serial dilution series of a concentrated stock of bovine serum albumin (BSA) (Sigma-Aldrich Co., PN-A3294) in MilliQ™ water (Millipore Corporation). Micro-volume data was obtained with undiluted standard samples using the Take3 Multi-Volume Plate on the Epoch™ Microplate Spectrophotometer (BioTek Instruments, Inc.). The multi-volume plate was calibrated prior to use to determine pathlength correction values for each microspot location. Each standard was loaded five times at each microspot location on the 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.

Standard 1 cm pathlength data was acquired using a 1 cm vertical pathlength cuvette, the BioCell™ (BioTek Instruments, Inc.) and either undiluted standard samples for the lower concentration range or after 1:20 dilution in MilliQ water for higher concentrations. Dilutions were verified by mass analysis on an analytical balance. 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. Data analysis was performed using Gen5™ Data Analysis Software (BioTek Instruments, Inc.) and Excel® spreadsheet software (Microsoft Corporation).

Limit of Detection

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 multi-volume 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 made by mixing 80 µL BCA working reagent with 4 µL protein standards and samples (20:1 ratio) in microtubes. Samples were incubated at 37° C for 30 minutes, then 2 µL of each were loaded onto the Take3 plate in duplicate. The absorbance of each standard and sample was read at 562 nm with a microplate spectrophotometer.

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

Nucleic Acid Absorbance at 260 nm

Linear Dynamic Range

All double-stranded DNA (dsDNA) and RNA standards were created by preparing a 1:2 serial dilution series of a concentrated stock of herring sperm dsDNA or yeast (*Saccharomyces cerevisiae*) RNA, respectively, in TE buffer (tris-EDTA, pH=7.0). Micro-volume data was obtained with undiluted standard samples using the multi-volume plate. The plate was calibrated prior to use to determine pathlength correction values for each well location. Each standard concentration was loaded five times at each microspot location on the multi-volume plate using an 8-channel manual pipettor. Optical densities were read at 260, 280 and 320 nm resulting in 80 replicate measurements. Standard 1 cm pathlength data was acquired with a BioCell, using both undiluted low concentration samples and 20-fold dilutions of the higher concentration samples, diluted in TE or MilliQ water. All sample measurements were background corrected using a TE buffer blank or MilliQ water, where appropriate. All concentrations depicted are based on a 1 cm pathlength and 50 ng/mL/OD for DNA; 40 ng/mL/OD for RNA.

Limit of detection was calculated as described above for protein determinations with the exception that the primary wavelength was 260 nm.

Results & Discussion

Native Protein Absorbance at 280 nm

Linear Dynamic Range

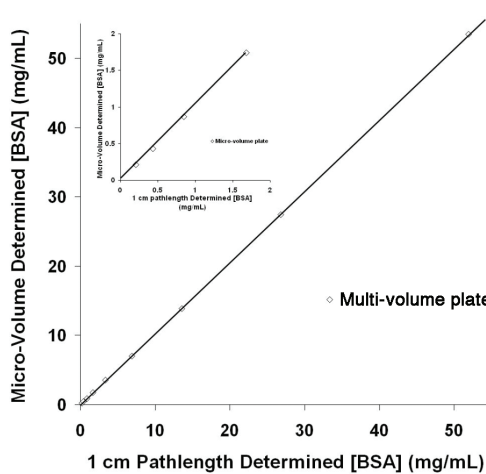


Figure 2 – Protein standard curve using dilutions of bovine serum albumin measured at 280 nm. Abscissa data is considered actual [protein] as measurements were performed with 1 cm pathlength in a vertical cell. Ordinate data is micro-volume determinations using the Take3 Multi-Volume Plate. Inset is exploded view of low protein concentration. In both graphs, the line fit represents a slope of 1.000 and demonstrates equivalence between micro-volume and 1 cm pathlength determinations.

- Linear dynamic range spans more than three orders of magnitude
- Covering concentration range from approximately 0.2-50 mg/mL
- Linear regression analysis $R^2 = 1$ and slope = 1.03
- Slope indicates an average percent accuracy of 3% relative to 1 cm pathlength determination

Precision

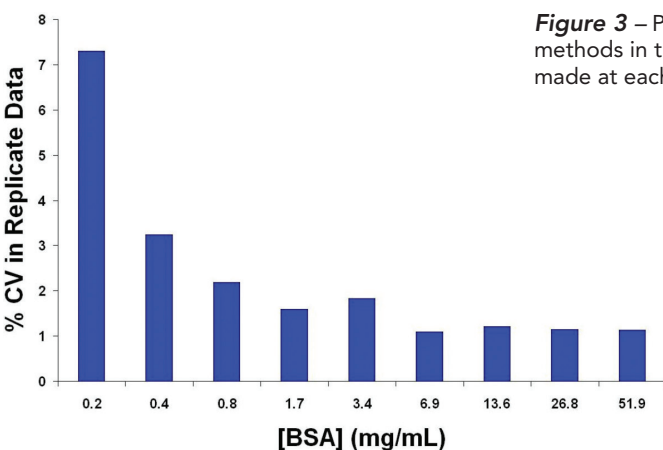


Figure 3 – Precision of micro-volume methods in the replicate measurements made at each BSA standard.

- Precision expressed as %RSD $\leq 2\%$ for [protein] ranging to approximately 0.5 mg/mL
- Precision degrades at lower concentrations

Limit of Detection

- Calculated limit of detection equals 0.0021 OD from blank measurements
- Average background corrected absorbance signal by 16 microspots was determined to be 0.078 OD
- Limit of detection for BSA is 6 µg/mL

Results & Discussion (cont.)

BCA Colorimetric Assay

Improved Sensitivity Relative to A280 measurements

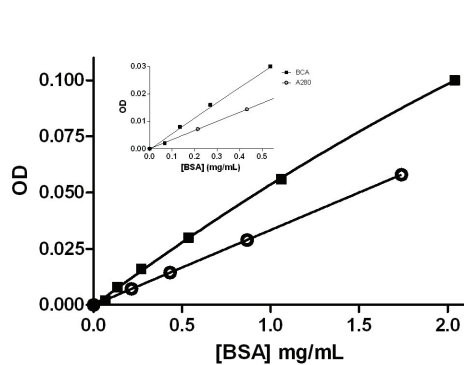


Figure 4 – Comparison of micro-volume standard curves using BCA (OD measurements at 562 nm) and A280 native protein absorbance using Epoch and Take3. The BCA calibration curve was fit with a quadratic equation as specified by the kit manufacturer; the A280 calibration curve used linear regression. Inset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

- Improved analytical sensitivity obtained by using BCA reagent
- BCA best fit with a quadratic equation; A280 data linear ($R^2 = 1.0000$)
- BCA provides greater accuracy due to:
 - Greater slope of the calibration curve
 - Relative freedom from interfering signals such as nucleic acids
- A280 analysis is a far simpler workflow that does not require the purchase and addition of colorimetric reagents

Native Nucleic Acid Absorbance at 260 nm

Linear Dynamic Range

dsDNA

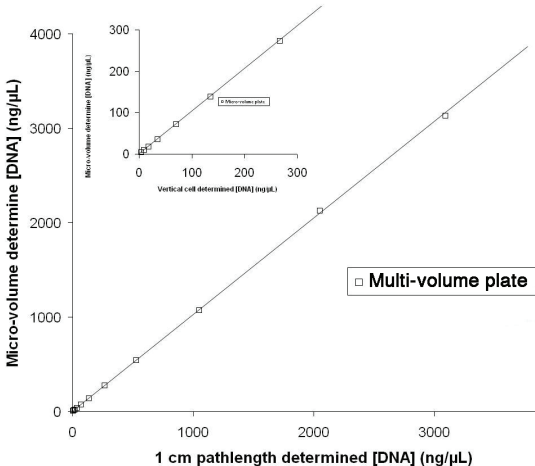


Figure 5 – dsDNA standard curve using dilutions of a purified herring sperm sample. Abscissa data is considered actual [dsDNA] as measurements were conducted with 1 cm pathlength using the vertical cell. Ordinate data is micro-volume determinations using the Take3 Multi-Volume Plate. The straight line is through the origin and has a slope of 1.000, which is considered a perfect equivalence between micro-volume and vertical cell data. Inset is an exploded view of low dsDNA concentrations.

- Linear dynamic range spans more than three orders of magnitude
- Covering a concentration range from approximately 4-3,000 ng/µL
- Linear regression analysis $R^2 \geq 0.9998$ and slope of 1.02
- Slope indicates average percent difference of 2% relative to 1 cm pathlength determinations

Results & Discussion (cont.)

RNA

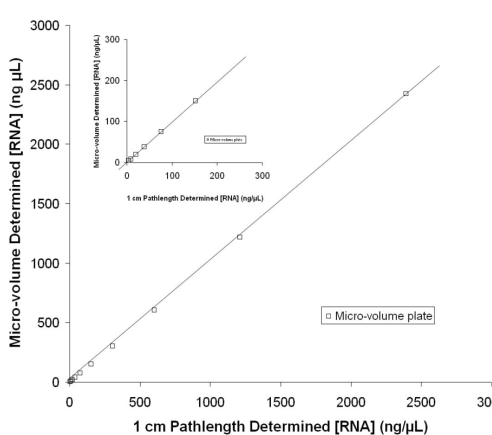


Figure 6 – RNA standard curve using dilutions of a purified yeast RNA sample. Abscissa data is considered actual [RNA] as measurements were conducted with 1 cm pathlength using a vertical cell. Ordinate data is micro-volume determinations using the Take3 Multi-Volume Plate. The straight line is through the origin and has a slope of 1.000, which is considered a perfect equivalence between micro-volume and 1 cm pathlength data. Inset is an exploded view of low RNA concentrations.

- Linear dynamic range spans three orders of magnitude
- Covering a range from approximately 4-2,400 ng/µL
- Linear regression analysis $R^2 = 1.00$ and slope of 0.9990
- Slope is indicative of an average percent difference of 1% relative to 1 cm pathlength determinations

Precision – dsDNA and RNA

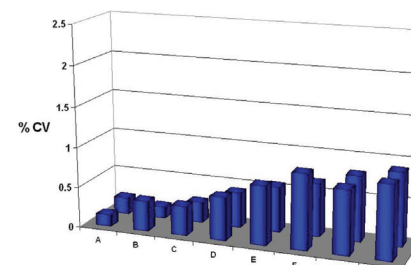


Figure 7 – Precision measurements expressed as %CV from each of the individual 16 microspots at dsDNA concentrations of 266 ng/µL.

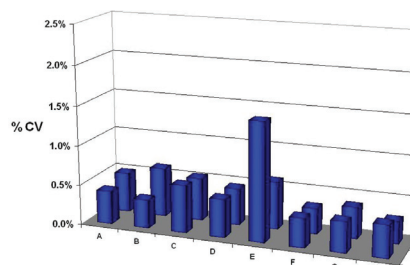


Figure 8 – Precision measurements expressed as %CV from each of the individual 16 microspots at RNA concentrations of 304 ng/µL.

Limit of Detection

- Calculated limit of detection equals 0.0011 OD from blank measurements
- Average background corrected absorbance signal by 16 microspots was determined to be 0.0042 OD for dsDNA and 0.0053 OD for RNA
- Limit of detection for dsDNA and RNA are 1.2 and 0.90 ng/µL, respectively

Conclusions

1. When using native UV absorbance the Take3 Multi-Volume Plate can provide accurate biomolecular analysis across a broad range of concentration spanning three orders of magnitude.
2. The use of BCA reagent provided the ability to characterize proteins across three orders of magnitude with significantly greater sensitivity when compared to native protein measurements as A280.
3. Detection limits of 6 µg/mL were observed when observing purified protein in MilliQ water at 280 nm and 1 ng/µL nucleic acids at 260 nm using the Take3 Multi-Volume Plate.
4. Precision was typically below 1% CV, but no more than 2.5% CV at low nucleic acid concentration and $\leq 2\%$ for protein ranging from high BSA concentrations to about 0.5 mg/mL.