



In-situ Micro-Volume Bicinchoninic Acid Protein Assay

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Standard protocols for bicinchoninic acid (BCA) assays using vials or microplates have been modified for use with the Take3™ Multi-Volume Plate suitable for micro-volume analysis. Protein samples and BCA working reagent are added sequentially directly to the microspots of the Take3 plate, incubated, then read on the Epoch™ Multi-Volume Spectrophotometer. Improvements in sensitivity are evident relative to standard protocols using 20:1 volume ratios of BCA working reagent to protein. Comparisons to a “Mini-BCA” assay workflow indicate that in-situ analysis is more accurate.

Introduction

Bicinchoninic acid (BCA) is one of the most popular reagents for colorimetric protein determinations. Many reagent vendors supply BCA kits for use with cuvette- or microplate-based spectrophotometers. The popularity of BCA stems from ease of use, stability and sensitivity relative to other colorimetric assays. Compared to native protein absorbance at 280 nm, BCA has improved sensitivity and good specificity for protein with minimal interference from nucleic acids, which is always a hazard when quantifying cell lysate or other biological samples. 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. Figure 1 demonstrates this reaction which is carried out in one step in most BCA protocols.

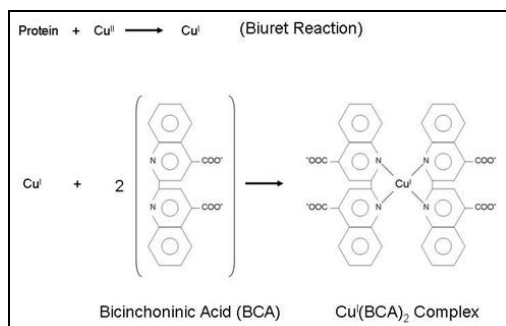


Figure 1. BCA reaction for protein quantification. $\text{Cu}(\text{BCA})_2$ complex has a strong molar absorptivity at 592 nm of 7,700 L/mol/cm [1].

Recently, micro-volume absorbance-based protein quantification using extremely short measurement pathlengths, have been demonstrated with dedicated instruments such as NanoDrop (ThermoFisher Scientific). This instrument and methods inherent are particularly useful using native protein absorbance at 280 nm due to sample conservation and ease of use. Protocols have also been developed for colorimetric reagents such as BCA. Using a modified protocol that calls for changes in the relative concentrations of protein and BCA working reagent from a 20:1 volume ratio of BCA working reagent to protein to a 1:1 ratio, micro-volume methods can shift the working range of the calibration curve relative to microplate- or cuvette-based measurements. This is called a “Mini-BCA” assay by the NanoDrop manual [2]. Yet for these pedestal-type micro-volume instruments, protein and BCA working reagent need to be mixed and incubated in a separate vessel before a small aliquot of the reaction mix is analyzed in the instrument, which reduces ease of use and the ability for sample conservation.

Here we show the utility of the Take3 plate for in-situ micro-volume BCA assays mixed and incubated in the microspots of the plate. The assay workflow is simple and resembles the workflow for a typical microplate assay where reagents and samples are added sequentially to microplate wells without the need for offline color development. This significantly improves ease of use. Further, as only 2 μL of sample is consumed, this promotes sample conservation.

Material and Methods

Bicinchoninic Acid Kit and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. The BCA assay was demonstrated using two micro-volume formats. The first format was the “Mini-BCA” assay recommended by the NanoDrop technical manual [2]. Briefly the BCA assay comprised mixing 10 μL BCA working reagent with 10 μL protein standards and samples (1:1 ratio) in microtubes, incubations were performed at 37° C for 30 minutes, cooled to room temperature for 15 minutes and then 2 μL loaded into the Take3 plate in duplicate.

The second format was developed to allow in-situ analysis directly on the microspots of the Take3 plate (Figure 2). Briefly the BCA assay was made by adding sequentially 2 μL protein standards and samples, followed by 2 μL BCA working reagent directly onto the microspots. Protein standards and samples were run in duplicate and loaded with a single channel pipettor. This was followed by addition of 2 μL BCA working reagent using a multi-channel pipettor with mixing. Incubations were performed at room temperature (~ 22°C) for 25 minutes, unless otherwise noted.

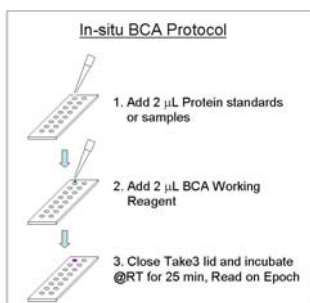


Figure 2. The 16 microspots of the Take3™ Multi-Volume Plate can serve as both reaction and detection vessels for in-situ micro-volume BCA assays. The assays are incubated at room temperature with the Take3 plate lid closed for 25 minutes before being read on the Epoch Spectrophotometer.

Bovine serum albumin (BSA) protein standards were prepared as either a 7 point 1:3 or 1:2 serial dilution series resulting in concentrations in the range of ~ 0.01 - 2 mg/mL or 0.01 - 3 mg/mL, respectively. The 1:2 serial dilution series was used for sensitivity comparisons between the two methods. For the comparative assessment of accuracy, a defined Take3 plate map was used according to Figure 3 using a 6 point 1:3 serial dilution series resulting in a working range of 0.01 - 1.0 mg/mL.

This format is recommended for all in-situ micro-volume analyses on the Take3 plate.

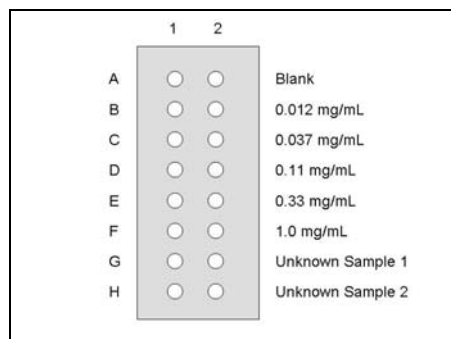


Figure 3. Take3 plate map depicting position and concentrations of BSA standards and positions of up to four unknown standards. This map provides a 6-point calibration curve run in duplicate over rows A-F, which covers the in-situ micro-volume assay working range of 0.01 – 1.0 mg/mL and 4 microspots for unknown samples over rows G-H. This map was used for both in-situ and “Mini-BSA” formats for the comparison of accuracy.

The blank was comprised of a 1:1 volume ratio of BCA working reagent and de-ionized water. All BCA assay measurements were made at 562 nm. Actual BSA concentrations in the *Improved Quantification Accuracy* section were made using underivatized BSA and native 280 nm absorbance measurements using the 1 cm vertical pathlength BioCell™ cuvette. All absorbance measurements were made using the Epoch Multi-volume Spectrophotometer system with Take3 plate. For all assays, measurements were made within 10 minutes of each other. Standard curves were fit using a quadratic function in GraphPad Prism.

Results & Discussion

Reaction Kinetics

Typical BCA protocols call for 20:1 volume ratios between BCA working reagent and protein samples [3]. The NanoDrop manual specifies 4 μL of protein diluted with 80 μL of BCA working reagent. Even at the high end of calibration curves (i.e. 2 mg/mL BSA), this is a vast molar excess of the active ingredients of the BCA working reagent to protein (Table 1). When compared to the number of possible reaction sites for the biuret reaction (peptide bonds, cysteine, cystine, tryptophan, tyrosine) however, this excess is narrowed, but protein 3° structure will limit access of Cu^{2+} and BCA to a large proportion of these sites. Thus reducing the dilution of the protein sample with BCA working reagent should maintain reaction kinetics and extend the working range of the calibration curve to lower protein concentrations.

[BSA] (mg/mL)	[CuSO ₄] (% w/w)	[BCA] (% w/w)	[Cu ²⁺]/[BSA] (mol L ⁻¹ /mol L ⁻¹)	[BCA] ₂ /[BSA] (mol L ⁻¹ /mol L ⁻¹)
2.0	4	1	2,100	8,500

Table 1. Concentrations and molar ratios between bovine serum albumin standard and the active ingredients of BCA working reagent for a 20:1 volume ratio of BCA working reagent to protein standard.

A 1:1 volume ratio of BCA working reagent to protein is the format for what the NanoDrop manual specifies as a “Mini-BCA” assay, where 10 μL of protein sample is reacted with 10 μL of BCA working reagent. This significantly reduces the dilution of protein sample and shifts the dynamic range of the BCA assay by about an order of magnitude to BSA concentrations of about 0.01 mg/mL [4].

We have modified this protocol for the in-situ micro-volume BCA assay by adding sequentially 2 μL of BSA standard or sample, followed by 2 μL of BCA working reagent to the microspots of the Take3 plate. Incubation was performed at room temperature with the Take3 plate lid closed. Figure 4 demonstrates the progress of the reaction with a 1 mg/mL BSA standard.

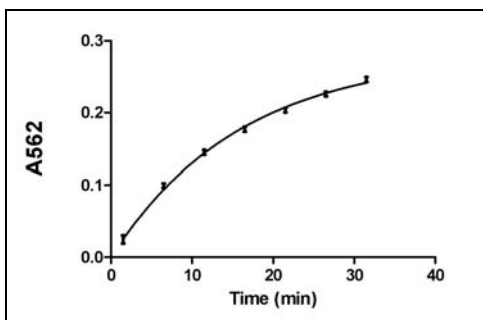


Figure 4. Reaction progress of the in-situ micro-volume BCA assay as measured colorimetrically at 562 nm. A BSA concentration of 1.0 mg/mL was used. Incubation temperature was ambient and measured to be 22°C.

Most BCA assay protocols require 2 hours for color development at room temperature or the use of elevated temperatures (37 or 60 °C) to reduce incubation time. Here we achieve satisfactory color development within 20 minutes. We selected an incubation time of 25 minutes for experiments going forward as a balance between maximizing color development and minimizing any effects from evaporation of the 4 μL reaction volume.

Sensitivity Enhancement using 1:1 Volume Ratio

Figure 5 demonstrates the added sensitivity available for in-situ analysis using a 1:1 volume ratio of BCA working reagent to protein sample compared to an assay incubated offline using a 20:1 ratio.

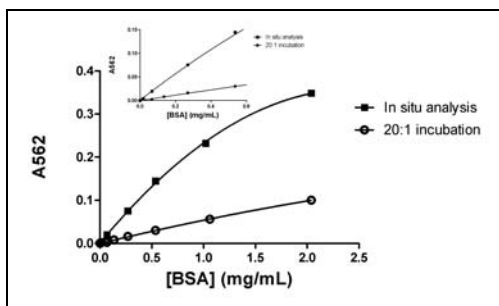


Figure 5. Take3 micro-volume analysis comparison between an in situ assay and one conducted in a micro-centrifuge tube using a 20:1 volume ratio of BCA working reagent to protein standard.

This gain in sensitivity, brought about by the significant reduction in protein dilution with the BCA reagent, extends the dynamic range to much lower protein concentrations to a limit of quantification of about 0.01 mg/mL BSA. It is evident that linearity is beginning to be compromised at BSA concentrations of ≥ 2 mg/mL compared to the 20:1 ratio. This is due to the molar excess of Cu^{2+} relative to BSA being reduced to about 100-fold. Considering there are over 650 possible reaction sites in the primary structure of BSA, the protein becomes the limiting reagent in the biuret portion of the reaction. This is validated by taking the calibration curve out to 3 mg/mL (data not shown) where a flattening of the curve occurs. For this reason, we limit the working range of the in-situ calibration curve to a concentration range between 0.01 – 1 mg/mL.

Improved Quantification Accuracy

The 16 microspots on the Take3 plate offers the ability to conduct in-situ micro-volume analysis of standards and unknowns all at the same time, much like in a conventional 96-well microplate. This can reduce errors in quantification accuracy due to incubation time differences and temperature fluctuations – variables known to influence the BCA assay [3]. Using the Take3 plate map defined in Figure 3, in-situ workflow was compared to the “Mini-BCA” assay. Results appear in Table 2. It is apparent that a significant improvement in accuracy is provided by using the in-situ workflow.

[Actual BSA] (mg/mL)	% Accuracy	
	In-situ	Mini-BCA
0.037	-0.99	-5.6
0.34	-1.5	-12

Table 2. Comparison of the accuracy in the measurement of two BSA samples using the in-situ and “Mini-BCA” workflows. Negative % accuracies depict an underestimation of BSA concentration. Conversely, positive % accuracies would represent an overestimation. Accuracies reflect n=3 experiments for each condition.

Conclusion

Both in-situ and “Mini-BCA” micro-volume assay formats shift the protein concentration working range down by approximately an order of magnitude relative to the standard BCA protocol that uses a 20:1 volume ratio of BCA working reagent to protein. In-situ BCA analysis provides a simpler, faster and more economical assay from the perspective of both protein and BCA working reagent relative to the “Mini-BCA” assay format, however. Furthermore, the in-situ format provides the ability to add standards and unknowns on the same microplate yielding greater quantification accuracy.

References

1. Brenner, A.J. and Harris, E.D. (1995). *A Quantitative Test for Copper using Bicinchoninic Acid*. *Anal. Biochem.* 226(1), pp. 80-84.
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Rev. 2/26/10, AN22610_07