Miniaturization and improved throughput of the BCA concentration determination method

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- **Successful miniaturization of absorbance assays is limited in part due to a short path length inherent in low volumes**
- **The fluorescent nature of white microplates was exploited in this epi-absorbance fluorescent readout**
- **As little as 1.5 µl of sample and 7.5 µl of reagent can be used in a 1536 well plate**

Introduction

Determining total protein concentration is an accepted means of comparing and standardizing biological samples. The utility of protein concentration assessment extends to high-throughput screening platforms including proteomic and genomic applications. Ideally the protein concentration determination should also be amenable to high throughput. Most protein determination assays use absorbance measurement detection which is difficult to minimize for higher throughput. Previously, it has been reported that colorimetric assays, including the bicinchoninic acid (BCA) protein assay, can be performed in white plates using fluorescence detection[1,2]. The method exploits the inherent fluorescence of white plates. In presence of an absorbing solution, the inherent fluorescence is quenched and the decrease in fluorescent signal can be used to measure colorimetric assays.

Assay Principle

The epi-absorbance approach described here relies on the fact that white plates exhibit fluorescent characteristics such that when an excitation light of ~ 435 nm is shined on the plate an emission signal at about ~ 560 nm can be observed (Figure 1). The colorimetric reagent/product absorbs light and suppresses the fluorescent signal that can be detected. Further development of the colorimetric product leads to a further decrease in fluorescent signal detected.

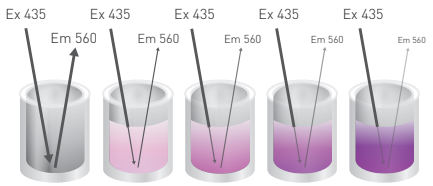

Fig. 1: Epi-absorbance principle for fluorescent detection of BCA. White plates have a characteristic fluorescent ex/em profile. This can be used in colormetric assays due to the ability of developed color to absorb light and thus amount of color change can be correlated to the intensity of fluorescent signal detected.

Materials & Methods

- Pierce™ BCA Protein Assay Kit and Pre-Diluted Protein Standards: Bovine Serum Albumin (BSA) Set (Thermo Scientific)
- White, low volume 384 well plates (Greiner/Corning)
- White, 1536 well plates (Labcyte)
- CLARIOstar® microplate reader (BMG LABTECH)

Spectral scanning
Empty wells and wells containing prepared BCA reagent with or without varying concentrations of BSA were added to each plate type. These were scanned with the CLARIOstar using the following settings:

	Excitation Scan	Emission Scan
Excitation wavelength [nm]	374 -> 534	417
Excitation bandwidth [nm]	10	16
Emission wavelength [nm]	562	445 -> 630
Emission bandwidth [nm]	16	10
Gain (Greiner 384)	1599	1254
Gain (Corning 384)	1506	1416
Gain (Labcyte 1536)	1290	996

Fluorescent detection of BCA Assay
Replicates of each BSA concentration were added to the plates. A volume of 2 µl was used for 384 well plates and a volume of 1.5 µl was used for 1536 well plates. Prepared BCA reagent was subsequently added. A volume of 10 µl was used in 384 well plates and 7.5 µl in 1536 well plates. The plates were read on the CLARIOstar with the settings indicated below.

Excitation	435 - 15
Dichroic	497.2
Emission	562 - 20
Gain (Corning)	1758
Gain (Greiner)	1729
Gain (Labcyte)	1407

Data were transformed to enable analysis with a linear regression fit.

$$OD_{562} = -\log(F/F_0)$$

F= Fluorescence, F_0 = buffer blank

Results & Discussion

The comparison of two low volume 384 well plates (Greiner and Corning) and a 1536 well plate (Labcyte) shows reasonably similar spectra for each plate with excitation maxima in the range of 435-450 (Fig 2 A) and emission maxima in the range of 460-510 (Fig. 2 B).

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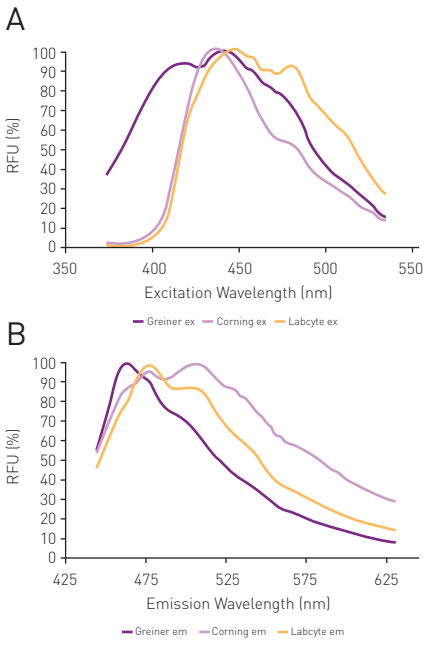

Fig. 2: Excitation and Emission Spectra for white microplates. Similar, but not identical excitation and emission spectra are observed for 384 well plates from Greiner ( ) and Corning ( ), as well as 1536 well plates from Labcyte ( )

BCA reagent decreases both, fluorescence excitation and emission peaks. A further decrease is observed in the presence of increasing protein concentrations following color development (Fig 3).

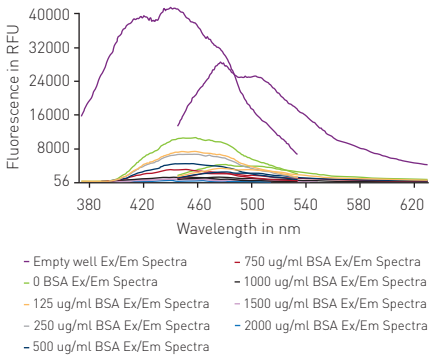

Fig. 3: Effect of BCA reagent and increasing concentration of BSA on excitation/emission spectra of white plates. BCA reagent alone suppresses the intensity of Ex/ Em spectra. Further suppression is observed with increasing concentrations of BSA. Representative data from Labcyte 1536 well plate is shown.

Based on this spectral data the CLARIOstar LVF monochromator™ was set to 435-15 for excitation and 562-20 for emission and the linear variable dichroic was automatically adjusted to 497.2 to read replicates of various concentrations of BSA (125-2000 mg/mL). The fluorescence measurements were used to create transformed standard curves, which, when graphed show that the 12 µl (2 µl protein) samples in 384 well plates conform to a linear fit as do the 9 µl (1.5 µl protein) samples in 1536 well plates (Fig. 4).

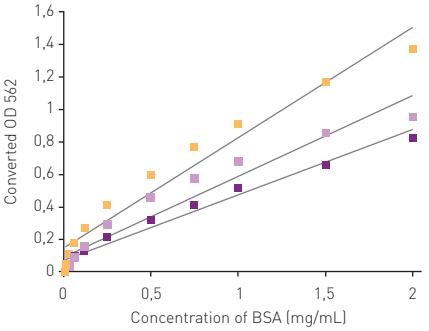

Fig. 3: Fluorescence data converted to OD_{562} for 3 plate types. Converted fluorescence data plotted based on their BSA concentration are shown. Data from each plate type can be fit using linear regression analysis. Low volume 384 well plates from Greiner ( ) exhibited an $R^2$of 0.973, Corning 384 well plates ( ) an R^2 of 0.934, and 1536 well plates from Labcyte ( ) an R^2 of 0.955.

Conclusion

Our results show that the epi-absorbance approach can successfully miniaturize the BCA assay. For the first time miniaturization to 1536 well plates is reported. Significant savings in reagent volume and sample volume can be achieved. Although the spectral characteristics of each plate differ slightly, these differences did not keep us from achieving direct comparisons that showed that all plates tested exhibit a good linear correlation between fluorescence data converted to OD_{562} and BSA concentration.

References

1. Zuck PI, O'Donnell GT, Cassady J, Chase P, Hodder P, Strulovci B, Ferrer M (2005) Miniaturization of absorbance assays using the fluorescent properties of white microplates. *Anal Biochem* **342** 254-259
2. Bainor AI, Chang L, McQuade TJ, Webb B, Gestwicki JE, (2010) Bicinchoninic acid (BCA) assay in low volume *Anal Biochem* **410** 310-312