

Old Assays, New Instrument: ELISA; NADH and NADPH Conversion; DNA and Protein Quantitation

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Application Note 169

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- Four classic absorbance assays using the Omega's high speed (~1 sec/well) UV/Vis spectrometer (220-850 nm)
- The FLUOstar/POLARstar/SPECTROstar Omega microplate readers can measure absorbance as never before
- Obtain 96 full UV/Vis spectra (220-850 nm) with a 1 nm resolution in less than 2 minutes

DNA Quantitation (260/280/320 nm)

Nucleic acids have an absorption maximum at 260 nm (A260) and spectrographic readings at this wavelength have become one of the most common methods for detecting DNA in a solution. Unfortunately, amino acids, single-stranded nucleic acids, and compounds used in the preparation of DNA can absorb at 260 nm, leading to abnormally high quantitation levels. However, these interfering compounds also absorb at 280 nm, leading to the calculation of DNA purity by performing ratio absorbance measurements at A260/A280. To be even more precise, background readings can be corrected by subtracting the reading at 320 nm. As a general rule any preparations with an A260-A320/A280-A320 greater than 1.7 is called "pure" DNA¹.

With the spectrometer from BMG LABTECH's Omega, the purity of DNA can be easily seen (Figure 1) because all wavelengths are given in less than 1 sec/well. The integrated path length correction and the new data analysis software, MARS, allow for fast determination of DNA concentration in samples (Figure 2).

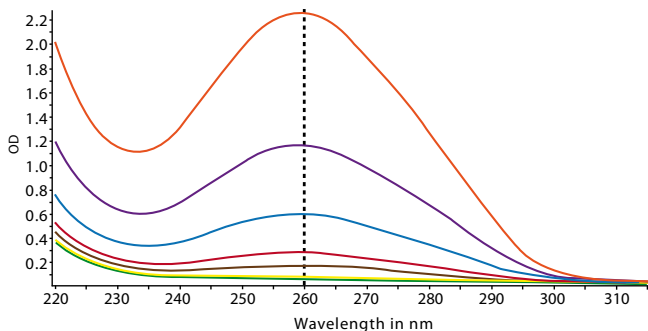


Fig. 1: Absorbance spectrum of different concentrations of calf thymus DNA recorded on the POLARstar Omega.

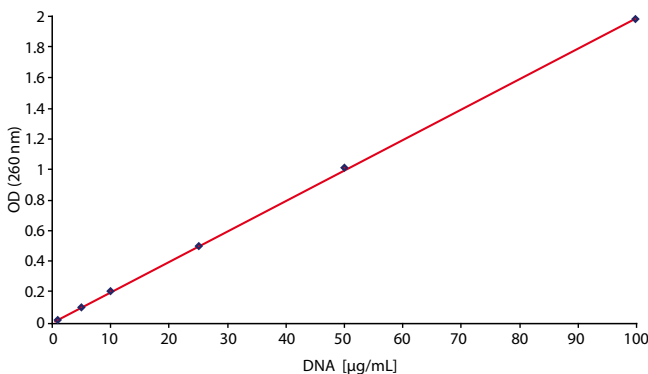


Fig. 2: Linear regression fit on the DNA standard curve in the concentration range from 0.1 to 100 µg/mL. An R²-Value of 0.99988 was obtained indicating a high degree of linearity throughout the range.

NADH/NAD and NADPH/NADP Conversion (340 nm)

NADH/NAD⁺ and NADPH/NADP⁺ are cofactors used by many enzymes in numerous cellular function, including: energy metabolism, mitochondrial functions, calcium homeostasis, oxidative stress, gene expression, immunological functions, aging and cell death².

The reduction of NAD⁺ to NADH and NADP⁺ to NADPH can be monitored at 340 nm because the oxidized forms do not absorb light at this wavelength. Figure 3 shows an absorbance spectrum of increasing amounts of NADH. Figure 4 is a linear regression fit of a NADPH/NADP concentration curve. Note there is an increasing linear relation at 340 nm and a decreasing linear relationship at 236 nm.

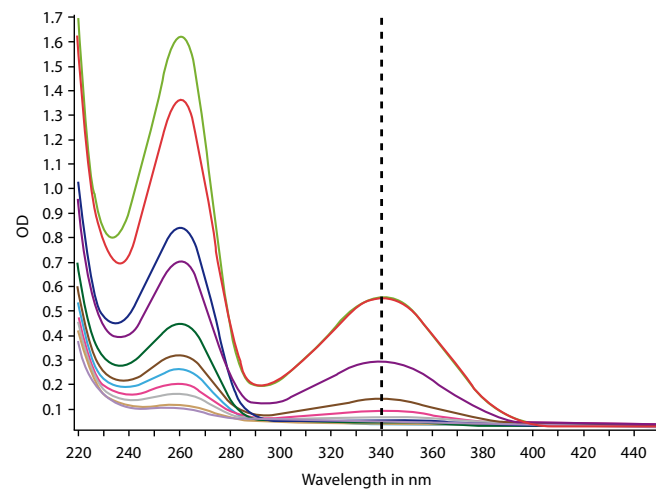


Fig. 3: Absorbance spectrum (220-450 nm) of different NADH concentrations. In contrast to NAD⁺, NADH shows an absorbance maximum at 340 nm. Similar results are obtained using NADPH.

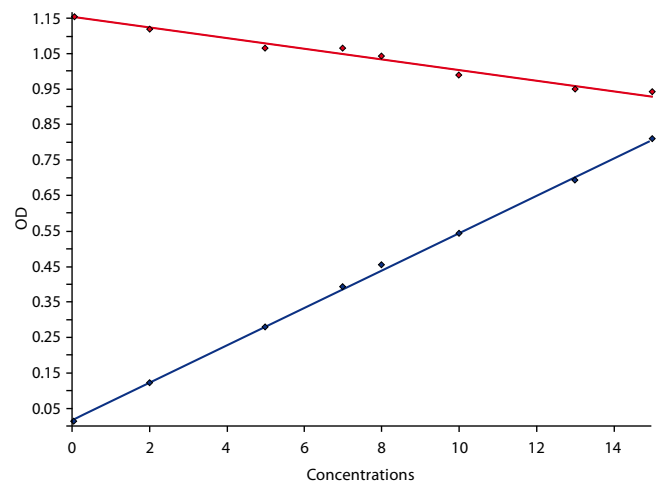


Fig. 4: Linear regression fit of the NADPH concentration curve at 340 nm (blue line, R²-Value of 0.9993) and 236 nm (red line, R²-Value of 0.975).

Quantification of NADH or NADPH allows for determination of enzymatic activity.

ELISA (405, 420, 450, 492, and 650 nm)

Enzyme Linked ImmunoSorbent Assay (ELISA) is a commonly used biochemical assay that can detect the presence of an antibody or an antigen in a sample. A sample is incubated with a secondary antibody that recognizes an antigen (or a primary antibody) and that is bioconjugated to an enzyme. This enzyme reacts with a substrate (which can undergo further reactions) producing a solution whose change in absorbance can be measured. The two most commonly used bioconjugated enzymes are horse radish peroxidase (HRP) and alkaline phosphatase (AP). AP's substrate, PNPP (p-Nitrophenyl phosphate), absorbs at 405 nm. HRP's substrate is hydrogen peroxide which is coupled with the following chromogens that can be measured with absorption spectroscopy: ABTS (2,2'-Azino-di-(3-ethylbenzothiazoline sulfonic acid)) (405-420 nm), TMB (tetramethylbenzidine) (650 nm or 450 nm), and OPD (O-phenylenediamine dihydrochloride) (492 nm). Mimicking an ELISA assay, yellow dye is used at varying concentrations (Figure 5). Linear regression fit of absorbance measurements at 450 and 430 nm (Figure 6) show that choosing the correct measurement wavelength can increase dynamic range significantly ($\times 13\%$).

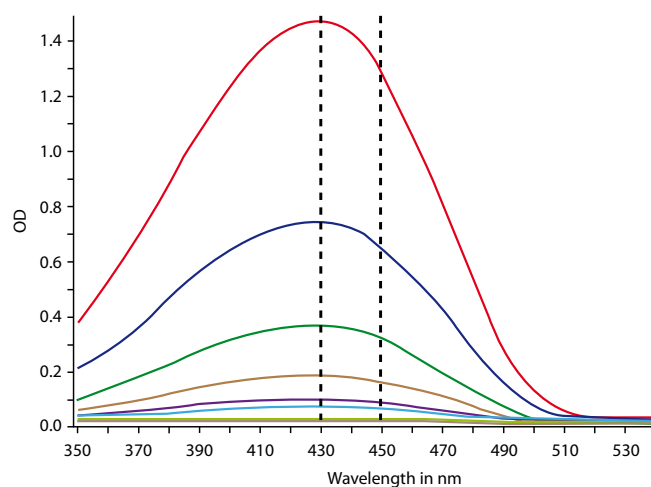


Fig. 5: Spectral absorbance curve of yellow dye which absorbs at similar wavelengths of most ELISA assays (400-500 nm).

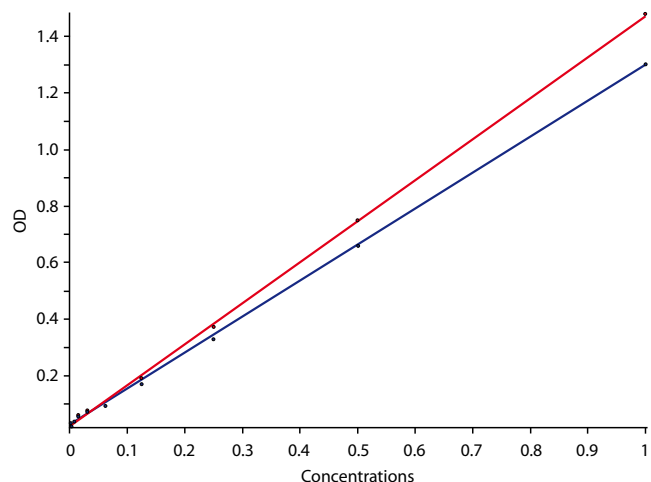


Fig. 6: Linear regression fit of OD measurements at 430 and 450 nm from figure 5.

References

- Ganske, F. (2008) Absorbance DNA Quantitation Using BMG LABTECH's POLARstar Omega Microplate Reader. BMG LABTECH application note 168.
- Dell, E.J. and Ganske, F. (2008) NADH and NADPH Conversion Monitored with BMG LABTECH's POLARstar Omega Microplate Reader. BMG LABTECH application note 170.
- Ganske, F. and Dell, E.J. (2007) Bradford Assay Performed on BMG LABTECH's FLUOstar Omega with new Evaluation Software. BMG LABTECH application note 158.

Bradford Protein Assay (595 nm)

Determining the protein concentration of samples is a necessary and often used method in biochemistry. Different colorimetric protein assays have been developed. The most commonly used methods are the Bradford assay (595 nm), the Lowry assay (790 nm) and the BCA assay (562 nm). The Bradford assay is based on the binding of protein to a dye (Coomassie® Brilliant Blue G-250), leading to a shift in the absorbance maximum of the dye from 465 nm to 595 nm (Figure 7)³. After creating a standard curve of protein solutions with known concentrations, the protein concentration of unknown samples can be calculated (Figure 8).

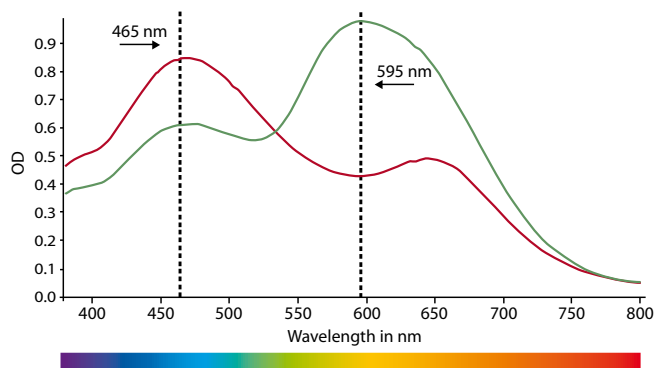


Fig. 7: The spectrum from unbound (red line) and protein bound (green line) Coomassie® Brilliant Blue. After binding the absorbance maximum of the dye shifts from 465 nm to 595 nm.

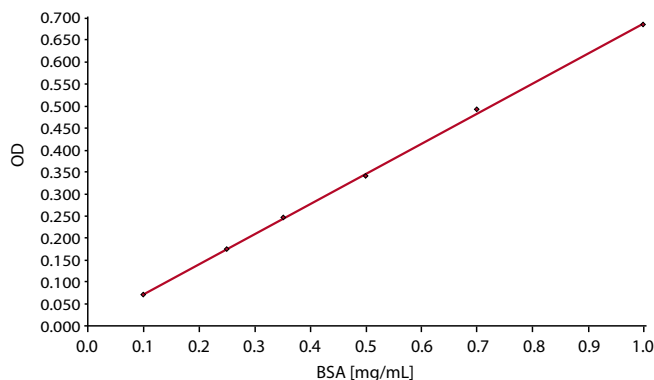


Fig. 8: BSA standard curve (linear regression fit performed with the MARS Data Analysis Software).

Conclusion

Four commonly used absorbance assays have been performed on the Omega microplate reader: ELISA, DNA and Bradford protein quantitations, as well as NADH and NADPH conversions. With the ability to read a UV/Vis spectrum (220-850 nm) at a resolution of 1, 2, 5, or 10 nm in about 1 second/well, the Omega can do absorbance assays never before imagined. The absorbance spectrometer along with the MARS evaluation software is currently available on the following BMG LABTECH microplate readers: FLUOstar, POLARstar, SPECTROstar Omegas.

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