Overview of ELISA assays and NADH/NADPH conversion detection

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- **Classic absorbance assays measured using spectrometer-based instruments**
- **Powerful MARS data analysis software utilized to increase assay window**
- **Obtain a full UV/Vis spectrum (220-1000 nm) in less than 1 second per well**

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 (Fig. 1)

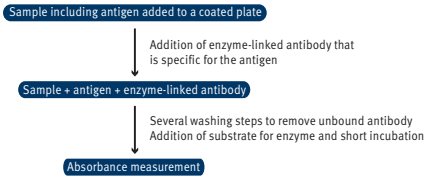

Fig. 1: ELISA Assay Principle.

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-ethylbenzthiazoline sulfonic acid)) (405-420 nm), TMB (tetramethylbenzidine) (650 nm or 450 nm), and OPD (O-phenylenediamine dihydrochloride) (492 nm).

Instrument Settings

	SPECTROstar® *Nano*	SPECTROstar®/ FLUOstar®/ POLARstar® Omega	CLARIOstar®	PHERAstar® *FS*
Detection mode	Absorbance			
Method	Endpoint			
Optic settings	Select either a full spectrum (220 – 1000 nm) or Select part of a spectrum (350-530 nm) or Select ELISA specific and reference wavelengths, e.g. 405 nm, 450 nm, 492 nm and 650 nm			

The BMG LABTECH´s control software offers pre-defined assay protocols. Just one click is needed to start the measurement.

Mimicking an ELISA assay, yellow dye is used at varying concentrations (Figure 2).

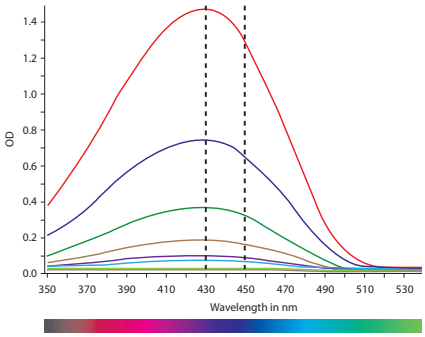

Fig. 2: Spectral absorbance curve of yellow dye which absorbs at similar wavelengths as most ELISA assays (400-500 nm).

Linear regression fit of absorbance measurements at 450 and 430 nm (Figure 3) show that choosing the correct measurement wavelength can increase dynamic range significantly (>13 %).

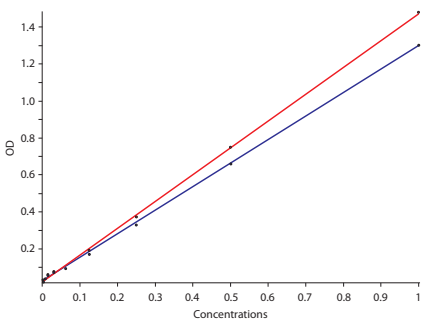

Fig. 3: Linear regression fit of OD measurements at 430 and 450 nm from Fig. 2.

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,

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immunological functions, aging and cell death. 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 (Fig. 4)

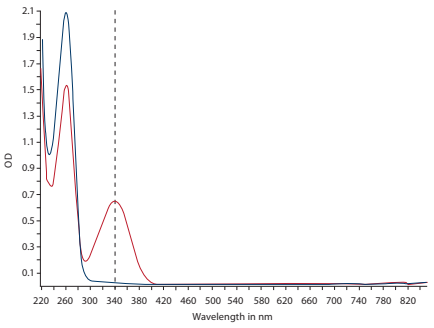

Fig. 4: Absorbance spectra of NAD^+ (blue line) and NADH (red line).

Instrument settings

	SPECTROstar *Nano*	SPECTROstar/ FLUOstar/ POLARstar Omega	CLARIOstar	PHERAstar *FS*
Detection mode	Absorbance			
Method	Endpoint			
Optic settings	Select either a full spectrum (220 – 1000 nm) or Select part of a spectrum (220-400 nm) or Select NADH/NADPH specific wavelength at 340 nm			

Using the MARS Data analysis software, linear regression fits of measurements taken at 260 and 340 nm can be done for $NADP^+$/NADPH or NAD^+/NADH conversion curve (Figure 5). A linear increase in signal at 340 nm is expected (example in Fig. 5 lead to a R^2=0.99). However, unlike the fit of a usual dose response curve, the linear regression fit at 260 nm shows a slight decrease in signal as the NADPH concentration increased (Fig. 5, red line, R^2=0.93). Theoretically, this peak should have no slope since the concentration of the dinucleotide is kept constant. However, there may be a slight decrease because it seems that $NADP^+$ absorbs more light at 260 nm than NADPH. A linear regression fit of the ratioed measurements (340/260 nm) (green line) can be done to correct for this change.

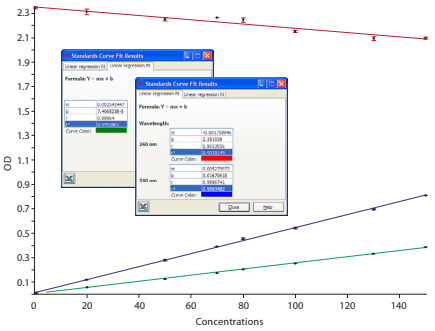

Fig. 5: Linear regression fit of absorbance measurements taken at 260 and 340 nm for a $NADP^+$/NADPH conversion curve. The red line is the fit of measurements taken at 260 nm, the blue line at 340 nm and the green line the ratio of the measurements taken at 340/260 nm.

In Table 1 the sensitivity for NADH and NADPH is shown depending on the number of flashes.

		NADH	NADPH
	Flashes	LOD µM (ng/mL)	LOD µM (ng/ml)
96 well	20	0.748 (497)	1.93 (1610)
	50	0.580 (386)	1.58 (1322)
	100	0.465 (309)	1.11 (931)
384 well	20	3.761 (2501)	1.80 (1497)
	50	3.418 (2773)	1.38 (1151)
	100	3.430 (2281)	1.40 (1169)

Conclusion

The microplate readers from BMG LABTECH all have a UV/Vis spectrometer that can measure any absorbance range from 220-1000 nm at 1, 2, 5 and 10 nm resolution in under 1 second per well. With this flexibility and speed, absorbance assays can be performed easily and fast.

In this application note we have shown the power of the spectrometer in measuring ELISA assays or assays that need the cofactors NAD^+, NADH, $NADP^+$ and NADPH.

PHERAstar® *FSX*

*The PHERAstar *FSX* is the newest PHERAstar reader.

CLARIOstar®

Omega Series

SPECTROstar® *Nano*