APPLICATION NOTE

Advantages of wavelength scanning in fluorescence and absorbance assays.

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EnVision™ XCite multimode plate reader

Introduction

A monochromator is an optical device known for its ability to separate the colors of light using an element called diffraction grating. By changing the angle of this grating the light exiting the monochromator through a fixed slit will be of a desired wavelength. This easy way of acquiring and selecting pure – or *monochromatic* – light makes it a suitable technology for microplate readers. Not only does it free users to experiment with assays regardless of their set of filters available, but it also facilitates some new application areas traditionally not associated with plate readers e.g. spectral scanning.

The EnVision XCite multimode plate reader, widely used in biological assay studies across the field and available in a number of configurations, incorporates monochromator technology in its two options. **Absorbance monochromator** option is capable of performing absorbance assays while **Fluorescence Intensity monochromator** option can be used for both absorbance and fluorescence intensity assays (quad monochromator design).

The EnVision with monochromators enables the user to measure any chromophore or fluorophore at any wavelength in 0.1 nm increments within the operational wavelength range. The range in fluorescence measurements is 230-850 nm, and up to 1000 nm in absorbance assays. The different spectral properties of a sample can be explored by inspecting the excitation, emission and absorbance graphs, while making use of the intuitive Start Wizard feature and various built-in calculations for data reduction. This application note demonstrates the use of EnVision in both absorbance and fluorescence assays using a common Bradford absorbance assay and a range of different fluorescent dyes.

Materials and methods

Absorbance assays

Bovine serum albumin (BSA, #A6003, Sigma) was dissolved in water to 2 mg/mL and a dilution series was prepared following Bio-Rad's standard microplate procedure (0-1500 µg/mL, 3 replicates). Protein Assay Dye (#500-0006, Bio-Rad) concentrate was prepared according to manufacturer's instructions for 1x Dye reagent. Assay was performed in clear 96-well SpectraPlate MB (#6005279, Revvity). The 1x Dye Reagent was added to wells with protein dilutions and the plate read with EnVision multimode plate reader (model 2104, Revvity) equipped with Fluorescent Intensity Monochromator option. The instrument protocol, with shake (30 s) and delay (5 min) operations was set before reading absorbance at 595 nm using both a filter and monochromator-based measurements. The plate map was defined with standard and blank sample types for standard curve fitting calculation (Fig. 1, Table 1). The Absorbance Wavelength scan protocol was performed for BSA standard samples (Fig. 2, Table 1) with a baseline correction prior the scan measurement. All data were analyzed with EnVision Manager (Revvity) or GraphPad Prism® (GraphPad Software Inc.).

Figure 1: Protocol structure and plate map. Plate is read with both filters and monochromators sequentially. The plate map defines the standard dilutions and their replicates (STD, red), blank samples (BL, yellow) and unknown concentration samples (UNK, light green).

Table 1: Overview of the assay-related settings in EnVision

	Exc.	Em.	Step	Flash no.	Operations	Calculations
BSA assay						
Absorbance scan	300 – 800 nm	—	1 nm	180	**Shake:** 30 s, 300 rpm, ø 0.3 mm, double orbital, inside	Curve fitting (blank corrected, std's on first plate only)
Mono meas.	595 nm	—	—	180	**Delay:** 300 s	**Fitting:** LinReg
Filter meas.	595/10 nm	—	—	10		
Fluorescein assay						
Excitation scan	400 – 550 nm	575 nm	0.5 nm	500		
Emission scan	425 nm	450 – 600 nm	0.5 nm	500		
GFP assay						
rGFP						
Excitation scan	300 – 530 nm	545 nm	1 nm	500		
Emission scan	425 nm	450 – 600 nm	1 nm	500		
EGFP						
Excitation scan	300 – 550 nm	565 nm	1 nm	500		
Emission scan	440 nm	460 – 600 nm	1 nm	500		
Quantum dot assay						
Absorbance scan	300 – 700 nm	—	1 nm	100		
Emission scan	300 nm	500 – 800 nm	1 nm	100		

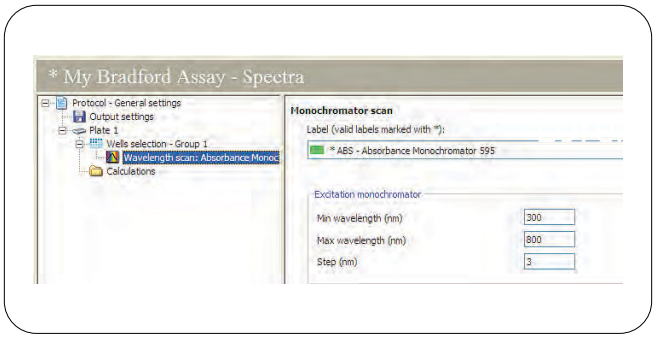

Figure 2: EnVision Manager settings for running a Wavelength Scan operation. The scan range and step size are entered in nanometres to the protocol. A baseline is required to be run through Wizard for absorbance wavelength scans.

Fluorescence assays

Fluorescein excitation and emission spectra were determined by using 50 µL of 100 nM Fluorescein standard solution (#C557-100, Revvity) in black 384-well OptiPlate (#6007279, Revvity). Both excitation and emission wavelength scans (Table 1) were read with EnVision and cuvette-based LS 55 spectrometer (Revvity). Recombinant wild-type GFP (rGFP, #8360-2, Clontech) and enhanced GFP (rEGFP, #8365-1, Clontech) were both diluted to 10 µg/mL. GFP samples (200 µL) were read for spectral properties (Table 1) with EnVision in black 96-well OptiPlate (#6005279, Revvity). Qdot® 655 (#Q10151MP, Invitrogen) was diluted to 25 µM, absorbance (with baseline correction) and emission wavelength scan (Table 1) read in EnVision using 96-well UV-Star® plate (#655801, Greiner) and black 96-well OptiPlate, respectively (200 µL).

Results

The absorbance spectrum of Bradford reagent is shifted as the BSA protein binds to Coomassie® (Imperial Chemical Industries, Ltd.) Brilliant Blue G-250 dye in increasing concentration. After overlaying the graphs from BSA standard wells, the shift in the absorbance can be seen in relation to the wavelength as the BSA concentration changes (Fig. 3). Binding to the BSA causes the Coomassie dye to shift its absorbance maximum from 465 to 595 nm. Also two isosbestic points can be defined at around 336 nm and 530 nm where the absorbance is equal irrespective of the BSA concentration. The baseline was recorded against a blank sample for the full absorbance range (230-1000 nm). This effectively corrects the absorbance values for drift that otherwise would occur during the scan due to the properties in light output of the Xenon flash lamp.

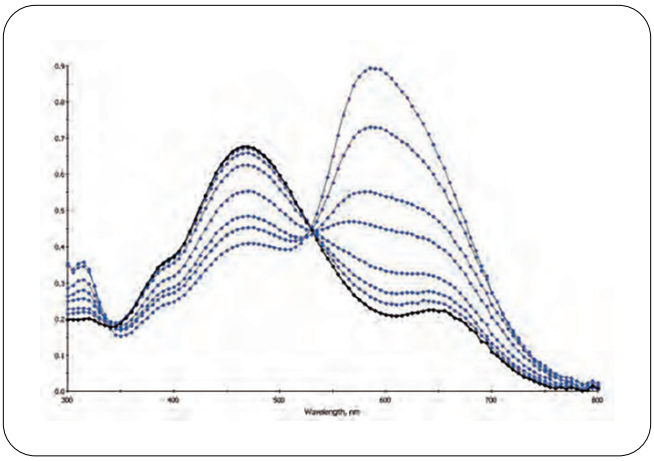

Figure 3: Changes in Coomassie® dye's absorbance properties. Several absorbance spectra overlaid as seen in the EnVision Manager software. Illustrated here are the BSA sample dilutions and their different absorbance properties.

The actual absorbance results were observed by comparing two standard sample plots, one measured with 595/10 nm photometric filter and other with monochromator set at 595 nm. The identical range and values outline the equal performance of monochromator option in absorbance assays (Fig. 4).

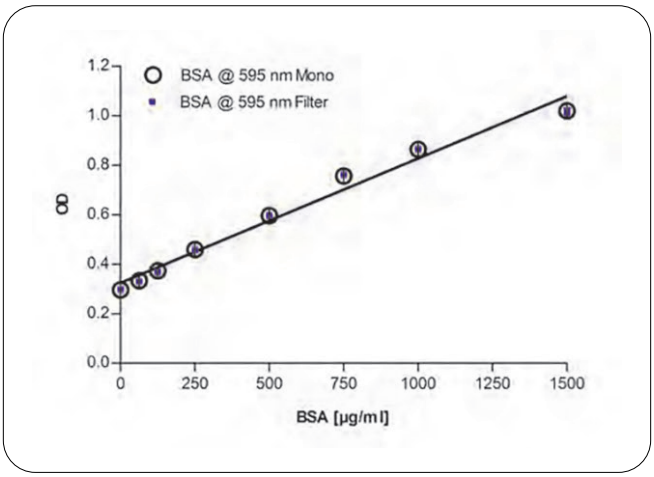

Figure 4: BSA standard concentration plot. The monochromator and filter measurement resulted in equal absorbance levels. The linear range of the assay for BSA is exceeded at 0.9 mg/mL, as seen from the linear fit. The Wallac EnVision Manager software can be used to exclude outliers and perform blank correction to the graph (data not shown).

The excitation and emission spectra for fluorescein were compared against the results obtained with a reference instrument, LS 55 Fluorescence Spectrometer. The acquired spectral profiles and peak wavelengths are well aligned with a peak excitation at 490 nm and emission at 513 nm (Fig. 5).

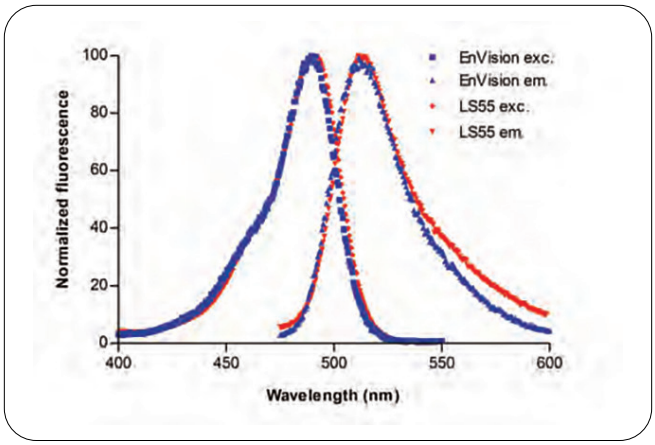

Figure 5: Fluorescein excitation and emission spectra as measured with EnVision and LS 55.

Green fluorescent protein (GFP) variants were examined for their differences in excitation and emission spectra. The two GFP variants show distinct differences in their excitation spectra while the emission properties are identical (Fig.6). The GFP in its wild-type form (rGFP) shows an excitation maximum at ~395 nm. The red-shifted form, EGFP, has its corresponding peak at ~485 nm increasing its excitation coefficient when used e.g. in confocal microscopy equipped with Argon-ion laser excitation (488 nm).

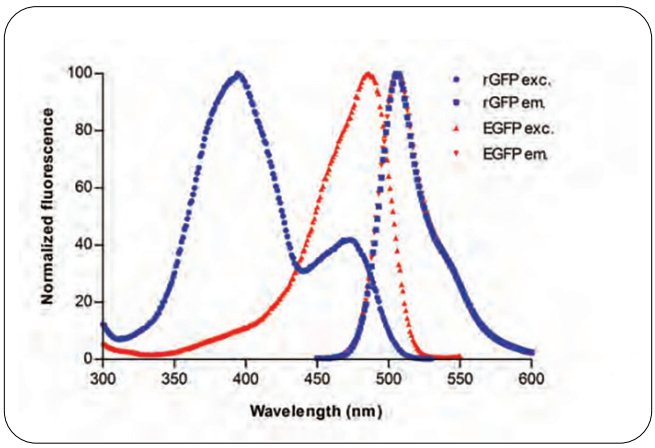

Figure 6: rGFP and rEGFP spectra. Two Aequorea victoria GFP variants were examined for their different excitation maxima. The excitation peak of EGFP is red-shifted to 485 nm, while the emission spectra maintains the same wavelength properties.

The absorbance and emission spectra for Qdot® 655 were determined using the EnVision Fluorescence Monochromator option in both absorbance and FI modes (Fig. 7). EnVision produced spectral data which also matches the reference absorbance and emission profiles (data not shown).

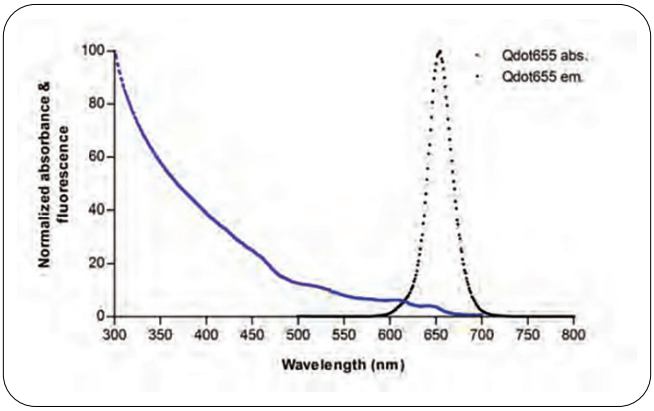

Figure 7: Absorbance and emission spectra for Qdot® 655 as measured with EnVision Fluorescence monochromator option.

Conclusions

EnVision is a flexible multimode plate reader that can be used in both HTS and basic research applications. The monochromator option expands the range of applications as it complements the suite of filters available. It will also help users to design assays for sensitive and fast filter-based measurements, perform spectral scanning and other more robust assays most suitable for a monochromator-based system. The data presented here outlines the accuracy and usefulness of the monochromator when applied to fluorescence and absorbance applications.

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