

The Importance of Spectral Scanning and Spectral Analysis for Achieving the Optimal Assay Performance

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Overview

This paper presents:

- how to gain advantages from using a spectral scanning microplate reader in assay optimization.
- how the reagent manufacturer's recommendations for measurement wavelengths can differ from the optimized wavelengths.
- how the assay environment can affect the label's spectral properties.
- how the measurement wavelengths of multi-label assays are optimized.

Introduction

Fluorometric measurement wavelengths are commonly selected following the reagent manufacturer's recommendations or taken from the literature. This does not necessarily lead to optimal assay performance, because:

- the reagent manufacturer's recommendations are not always based on the real peak excitation and emission wavelengths of the label.
- the peak excitation and/or emission wavelengths may shift according to the assay environment (e.g. pH, temperature, polarity and ionic strength)
- in a multi-label assay the optimal wavelengths depend on the selected label mixture and the effect of each label on each other.

Microplate readers can roughly be divided into two instrument types based on the technology used for the selection of measurement wavelengths: monochromator-based and filter-based instruments. Monochromator-based instruments use monochromators for wavelength selection, and can be used both for spectral and single/multi-wavelength measurements, as a full spectral range of wavelengths is always available onboard. Filter-based instruments use optical filters as wavelength selectors; so the wavelength range is limited to a certain number of filters available for the instrument model. Filter-based instruments cannot be used for measuring spectra. Monochromator-based instruments are, therefore, clearly superior to filter-based in optimizing the measurement parameters.

Methods

The measurement wavelengths of several fluorometric labels were optimized with a spectral scanning microplate reader. The materials used in this study were:

- from a commercial sourced Caspase-3 assay kit
- fluorometric labels: MeU, Alexa Fluor 488, 6-ROX, and Alexa Fluor 680
- 96-well microplates

The measurements were performed using the Thermo Scientific Varioskan Flash spectral scanning multitechnology microplate reader, which offers several measurement technologies (absorbance, fluorescence intensity, time-resolved fluorescence and luminescence). See Figure 1.



Figure 1. Varioskan® Flash spectral scanning multitechnology microplate reader

Results

1. Kit recommendations vs. optimal wavelengths

A commercial FRET (Fluorescence Resonance Energy Transfer) -based Caspase-3 assay was used to demonstrate that the measurement wavelengths recommended in the kit insert do not always represent the optimal

measurement wavelengths. The excitation and emission spectra of the label (AFC: 7-amino-4-trifluoromethyl coumarin) were measured to find the peak wavelengths, which were then compared to the wavelength recommendations of the kit insert. See Figure 2.

The kit manufacturer’s recommended wavelengths are 400 nm for excitation and 505 nm for emission. According to our spectral measurements, the actual peak wavelengths were 380 nm (excitation) and 490 nm (emission).

The measurement results show that:

- both excitation and emission maxima differed clearly from the wavelengths recommended in the kit insert.
- the signal level obtained with the 490 nm (=optimized) emission was ~10% higher than the signal level with 505 nm (= kit recommendation).
- the 380 nm excitation (= optimized) wavelength produced ~25% higher signals than 400 nm (= kit recommendation).

2. Assay environment

The fluorometric label MeU (methylumbelliferone) was used to demonstrate the effects of the assay environment on the fluorescence excitation and emission peak wavelengths and signal intensity. MeU fluorescence is strongly pH dependent; so the spectra were measured in varying buffer pH. See Figure 3.

This indicates that:

- pH affected both the excitation peak wavelength and the signal intensity of MeU.
- changes in pH did not shift the emission peak wavelength of MeU, but the signal intensity varied according to pH.

3. Multilabel assay

The effect of other labels on the optimal measurement wavelengths of 6-ROX was demonstrated in a multi-label assay of four fluorometric labels: MeU, Alexa Fluor 488, 6-ROX, and Alexa Fluor 680. The spectra of these labels spread to a wide wavelength area and, therefore, these labels are potential candidates for multiplexing assays. The spectra of each label were measured in a single-label solution and in a mixture of all the labels. See Table 1.

	Optimal measurement wavelengths for Varioskan Flash when used as single labels		Optimal measurement wavelengths for Varioskan Flash when used in a label mixture	
	Excitation (nm)	Emission (nm)	Excitation (nm)	Emission (nm)
MeU	360	448	358	446
Alexa Fluor 488	492	516	492	510
6-ROX	578	602	570	620
Alexa Fluor 680	677	704	677	702

Table 1. Optimized measurement wavelengths of the four labels, either as a single-label solution or in a multi-label solution, measured with the Varioskan Flash

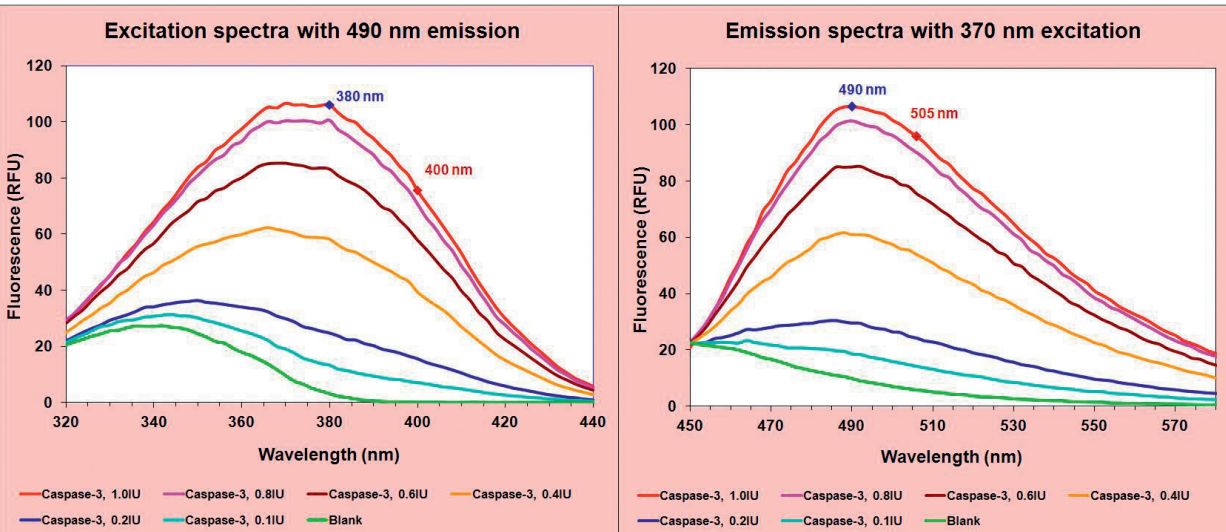


Figure 2. Excitation (left) and emission (right) spectra of the AFC label in varying caspase concentrations measured with the Varioskan Flash

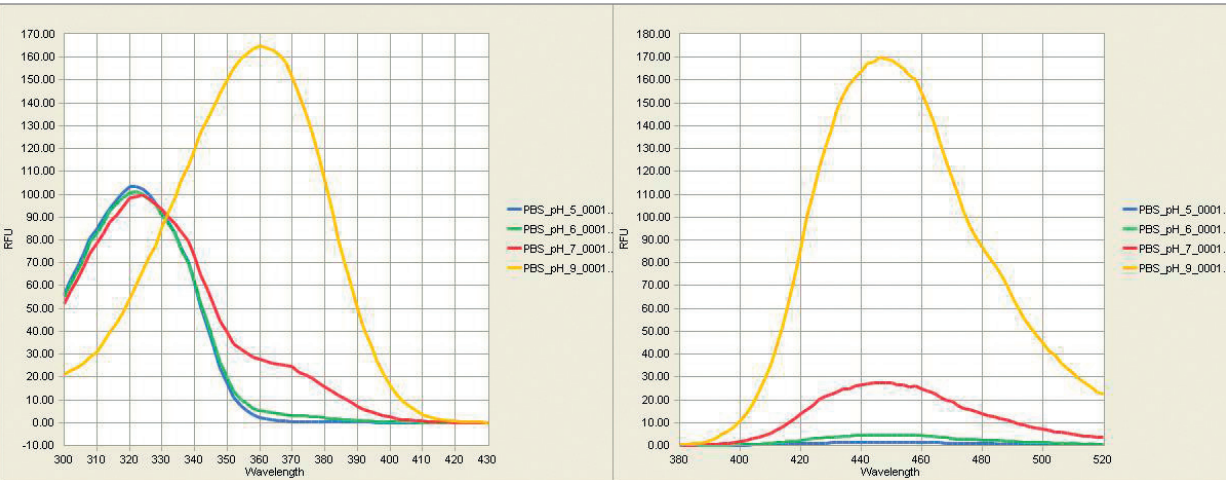


Figure 3. Left: pH dependence of MeU excitation spectra. Right: pH dependence of MeU emission spectra. Yellow = pH 9, Red = pH 7, Green = pH 6, Blue = pH 5. The optimal buffer pH for MeU was pH 9.

The measurements show that:

- optimal measurement wavelengths for the multi-label solution were quite similar to the optimal wavelengths of the single-label solutions for all the other labels except 6-ROX.
- in a multi-label solution 6-ROX had a ~20% higher signal/background ratio when using the optimized wavelengths for the label mixture in comparison to the wavelengths optimal for a single-label solution of 6-ROX.

Spectral analysis of the results

The Varioskan Flash microplate reader was controlled using Thermo Scientific SkanIt Software. This software offers an easy setup for the measurement of both excitation and emission spectra within the same protocol. The software includes automatic calculation options, e.g. for finding peak wavelengths, maximum signals and so on, thereby making the spectral analysis effortless. See Figure 4.

Conclusions

A spectral scanning instrument is very beneficial in:

- determining the real peak wavelengths of the spectra.
- further optimizing the performance of commercial kits instead of relying solely on the reagent manufacturer's recommendations.
- studying the spectral shifts according to changes in the assay environment.
- developing multi-label assays.
- checking the purity of reagents and samples.
- monitoring the stability of compounds or reagents.

For more information on the Varioskan Flash spectral scanning multitechnology microplate reader, visit: www.thermoscientific.com/varioskan

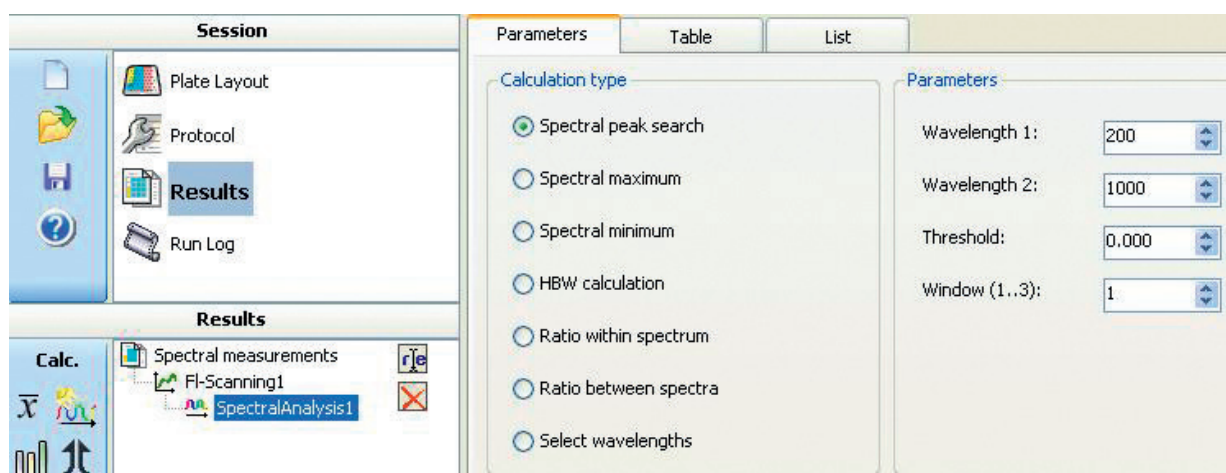


Figure 4. SkanIt® Software spectral analysis options make the result analysis quick and easy for assay optimization.

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