

# Optimization of FRET Assay with the Varioskan Spectral Scanning Microtiter Plate Fluorometer

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## Introduction

This paper presents the principle of optimization for fluorometric assays. Optimization is based on the measurement of fluorescence spectra with the Thermo Varioskan microtiter plate spectral scanning fluorometer. The optimal assay settings for the excitation and emission wavelengths are determined from this spectral data. The optimization procedure is shown with the FRET technology based QuantiCleave™ protease assay from Pierce Biotechnology Inc, Rockford, IL (product number 23267) and the ApoTarget Caspase-3 assay (product number KHZ0012) from BioSource International Inc, Camarillo, CA.

FRET (Fluorescence Resonance Energy Transfer) is an emerging fluorometric assay technology commonly used in proteomics. It is based on the use of two fluorometric labels, one acting as a donor and another as an acceptor molecule. When these molecules are in close proximity, emission energy of the donor is used for the excitation of the acceptor. With the long distance between the molecules the excitation of the acceptor is not possible. A common variation of this structure is to use one fluorometric label and one quencher that completely quenches the emission when in close proximity to the fluorescent label. In this case the fluorescent signal is detected when the quencher is removed.

QuantiCleave Fluorescent Protease Assay is based on a fluorescein-casein conjugate. The fluorescein label on the FTC-casein conjugate is highly quenched due to FRET based fluorescence homotransfer. The fluorescence which is observed in the homotransfer FRET mode occurs by way of electronic energy transfer between identical fluorophores. In this system, fluorescein acts both as the energy donor and acceptor. Protease activity is evidenced by an increase in fluorescence as the FTC-casein conjugate is digested by the protease. Fluorescence quenching is relieved and an increase in fluorescence is observed.

ApoTarget Caspase-3 protease assay is based on the recognition of the DEVD (Asp-Glu-Val-Asp) amino acid sequence linked to the fluorometric label AFC (7-amino-4-trifluoromethyl coumarin) by Caspase-3. The reaction substrate, DEVD-AMC, does not show the typical fluorometric emission with the linked peptide, but the fluorescence is restored when AFC is cleaved off the DEVD peptide. The resulting fluorescence is quantified and is proportional to Caspase-3 activity in the sample.

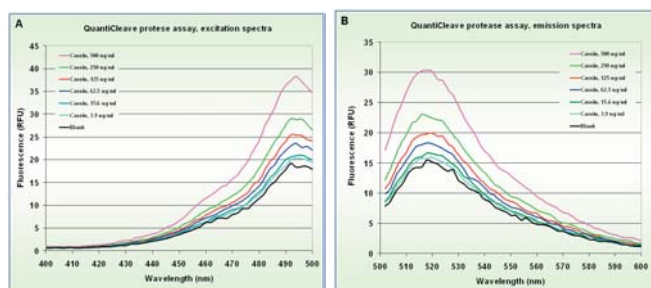


Figure 1. Varioskan excitation and emission spectra of FRET protease activity assay using QuantiCleave FTC-Casein as a substrate with different trypsin concentrations.

## Material and Methods

Both kits and reagents were used according to the instructions of the manufacturer with the following modifications:

In the QuantiCleave assay, TPCK trypsin was used as a sample in all tests. A dilution series between 0.2 - 500 ng/ml of TPCK Trypsin was prepared and 50 µl of each dilution was added into a black 384-well microtiter plate (Thermo Microtiter). Then 50 µl of FTC-Casein was added and the protease reaction was followed using kinetic reading from the beginning of the reaction. The instrument was set to read kinetic excitation and emission spectra every 15 minutes up to one hour. Spectral scanning settings were as follows: Read 1, fixed excitation 485 nm, excitation slit 5 nm, integration time 100 ms, emission spectra from wavelength area 500 - 600 nm, wavelength step 2 nm. Read 2, fixed emission, 517 nm, excitation spectra from wavelength area 400 - 500 nm, excitation slit 5 nm, integration time 100ms, wavelength step 2 nm.

In the Caspase-3 assay, a purified recombinant human Caspase-3 from BioSource (product number PHZ0014) was used as a sample. A dilution series from 0.1 to 1.0 IU of Caspase-3 in PBS was prepared and 50 µl of the dilution was transferred into the wells of a black 96-well microtiter plate (Thermo Microtiter). Then 50 µl of the fluorometric Caspase-3 substrate DEVD-AFC was added. Both emission and excitation spectra from the wells were measured after a 2-hour incubation. Excitation spectra were measured between 320 - 440 nm using fixed emissions at 490 nm and 505 nm. Emission spectra were measured between 400 - 580 nm using fixed excitations at 370 nm and 400 nm. These excitation and emission spectra were measured from all different Caspase-3 dilutions and from all time points.

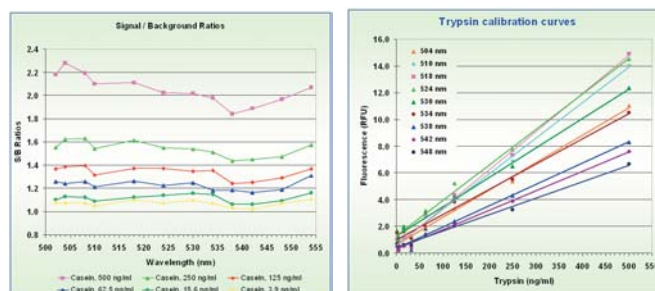


Figure 2. Signal to background ratios of QuantiCleave FRET protease activity assay according to different emission wavelengths. Figure 3. Trypsin calibration curves with different emission wavelengths in the QuantiCleave FRET protease assay. The assay blank has been subtracted from all values.



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## Results and Discussion

Excitation and emission spectra of FTC-Casein cleaved by the trypsin protease are shown in Figure 1. These spectra were measured using a 538 nm emission wavelength for the excitation spectrum and a 485 nm excitation wavelength for the emission spectrum, as recommended for the FTC-Casein substrate. As seen, both excitation and emission maxima are clearly different from the recommended excitation and emission wavelengths. The most efficient excitation is achieved with the 494 nm excitation wavelength and the maximal emission is located at 518 nm.

Based on the spectra, a further spectral analysis was performed with a fixed 494 nm excitation and only the effect of the different emission wavelength was further tested. This was done because the recommended excitation wavelength 485 was quite close to the maximum excitation efficiency (83%) when with the emission wavelength, only 60% of the maximum emission was achieved. Signal to background ratios with different emission wavelengths were determined with different trypsin concentrations and the results are shown in Figure 2. It is clearly seen that higher signal to background ratios are achieved in the wavelength area of 500 - 520 nm compared to the 530 - 550 nm wavelength area.

Finally, calibration curves of trypsin concentration were determined with different emission wavelengths. These calibration curves are collected into Figure 3. Based on this figure, optimal wavelengths for this assay can be determined. The strongest dose response is clearly achieved with 518 and 524 nm emission and that is in concordance with the location of the emission peak (see Figure 1.). As a result, the optimal detection wavelengths for this FRET-based fluorescent protease assay are based on this study excitation wavelength at 494 nm and the emission wavelength at 518 nm.

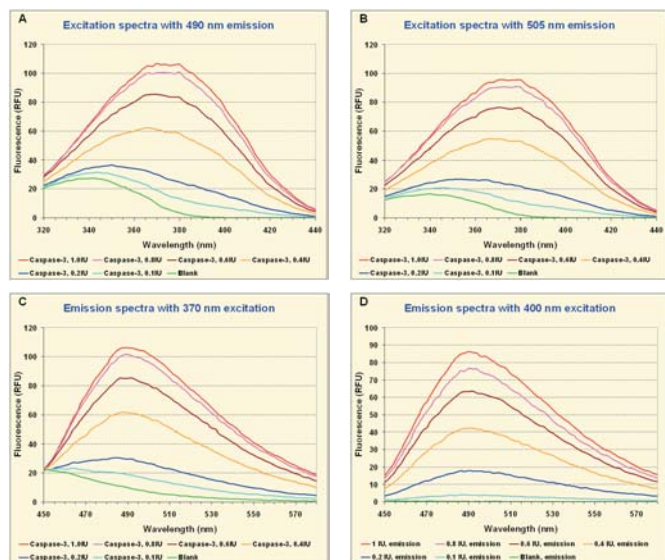


Figure 4. Excitation and emission spectra of the Caspase-3 assay with DEVD-AFC. Figures 1A and 1B show excitation spectra with two different fixed emission wavelengths and Figures 1C and 1D show emission spectra with fixed excitation wavelengths.

The four measured spectra of the Caspase-3 protease reaction with DEVD-AFC are shown in Figure 4. The most important findings from these figures are that both the excitation and emission maxima are clearly different from the wavelengths normally recommended for this fluorometric label. AFC is commonly measured with 400 nm excitation and 505 nm emission, when the maximum values are somewhat lower, 370 nm for the excitation (Figures 4A and 4B) and 490 nm for the emission (Figures 4C and 4D). Another important finding from figure 4 is that the signal level obtained with the 490 nm emission is about 10% higher than the signal level obtained from 505 nm emission. Even a stronger difference is noticed from the figures of the emission spectra (Figures 4C and 4D) where the difference is almost 25% and the 370 nm excitation wavelength clearly gives higher fluorescence.

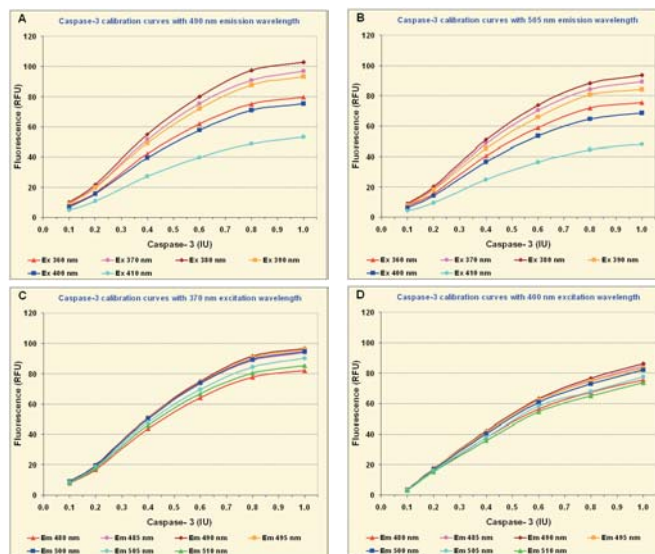


Figure 5. Caspase-3 calibration curves from different spectral scanings. Figures 2A and 2B present calibration curves with different excitation wavelengths with two emission wavelength and Figures 2C and 2D present the calibration curves with different emission wavelengths with two excitation wavelengths.

Calibration curves of Caspase-3 reaction were calculated from the spectral data for both fixed excitation and emission wavelengths. The resulting calibration curves are shown in Figure 5. It is clearly seen from the figures that the selection of excitation and emission wavelengths has a strong effect on the generated calibration curve.

With this Caspase-3 assay, the selection of excitation wavelength is more important than the selection of emission wavelength, because there are much stronger changes in the dose response when the excitation wavelength is changed than when the emission wavelength is changed. Based on figures 5A and 5B, the excitation wavelength producing the strongest dose response is 380 nm with both tested emission wavelengths, therefore being the optimal selection for the assay. The optimal emission wavelength can easily be selected based on Figures 5C and 5D, and the best choice would be 490 nm.

## Conclusions

Fluorometric assays have traditionally been performed with filter instruments and therefore commonly recommended excitation and emission wavelengths may well be based on the filter availability in addition to the spectral behavior of the label. Rather strong differences in assay performance can be obtained by using fluorescence spectral scanning for optimization of the excitation and emission wavelengths even with commercial fluorometric kits.

In the case of QuantiCleave FRET-based protease assay, the highest signals, better signal to background ratio, and therefore the best assay resolution and sensitivity were obtained with 494 nm excitation and 518 nm emission wavelengths instead of commonly used 485 nm excitation and 538 nm emission wavelengths.

With the fluorometric ApoTarget Caspase-3 assay, the best dose response was achieved using 370 nm excitation and 490 nm emission wavelengths instead of the 400 nm excitation and 505 nm emission wavelengths that are commonly used for the measurement of AFC.