Application Note

Microplate Instrumentation

Optimization of a time-resolved fluorescence resonance energy transfer (TR-FRET) assay for Varioskan®

Fluorescence resonance energy transfer (FRET) is a technique that permits the investigation of several different types of molecular, such as protein-protein, interactions. The energy transfer occurs over distances comparable to dimensions of biological macromolecules and is mainly used for homogeneous screening platforms.

This note will give an example of optimizing a TR-FRET assay for Varioskan® spectral scanning multimode reader, based on measuring fluorescence spectra and decay of the fluorescence.

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Abstract

Fluorescence resonance energy transfer (FRET) is a technique used to measure distance-dependent interactions on the scale of approximately 15-100 Å. It is based on usage of two different labels called donor and acceptor. The donor has to be fluorescent and the acceptor able to receive donor energy (Figure 1).
The donor is excited in the process and if the acceptor is in close proximity the excited state energy can be transferred. This leads to a reduction in the donor fluorescence intensity and excited state lifetime, and an increase in the acceptors emission intensity. Therefore there are several parameters to measure in order to detect energy transfer: change in donor intensity, lifetime and acceptor intensity. FRET includes no intermediate photon but the donor and acceptor are coupled by dipole-dipole interaction. In this particular assay the acceptor intensity is measured

Applications
- Measuring the distances or changes in distances in biologi cal systems (e.g. between two sites on a macromolecule)
- Protein-protein, protein-peptide and protein-drug interactions
- Conformational distribution in nucleic acids

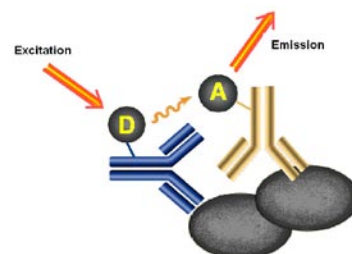

Figure 1. The antibodies bind to their epitopes on the target molecule and when the fluorophores are in close proximity the energy transfer is possible. The energy transfer leads to a decrease in donor fluorescence intensity and lifetime and an increase in acceptor emission intensity.

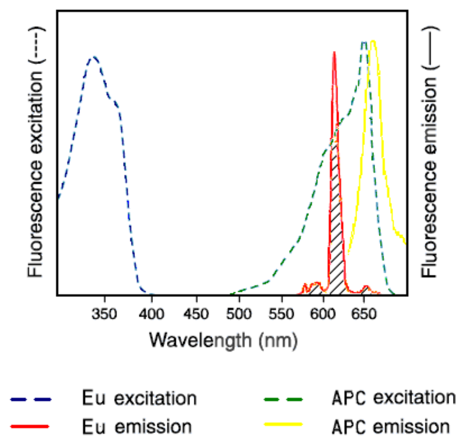

Figure 2. The excitation and emission spectra of the fluorophores in the assay. FRET occurs when the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor. In this figure the overlap is described as dashed.

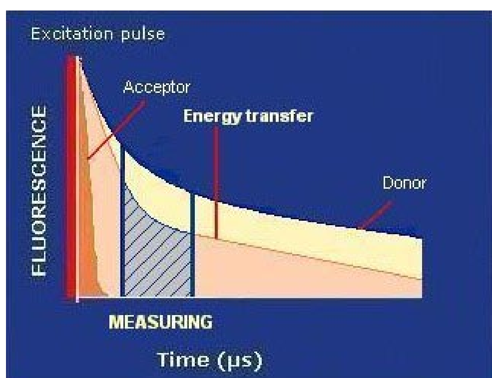

Figure 3. Basic principle of time-resolved FRET measurement. Because the donor fluorescence lifetime is longer than the acceptor lifetime and background, the energy transfer signal can be separated from the directly excited acceptor signal by using a suitable delay.

The efficiency of energy transfer strongly depends on the distance between the donor and acceptor molecules and on overlap of the donor molecule emission and acceptor molecule excitation spectra. (Figure 2.)
The transfer efficiency has an inverse 6th power dependency upon distance between the acceptor and donor. This makes the technique inherently sensitive to small physical changes in proximity. Mathematically the rate of energy transfer from a donor to an acceptor is following

$$k_T = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6$$

Where τ_D is the decay time of the donor in the absence of acceptor, R_0 is the Förster distance (the distance between the donor and acceptor where the average efficiency of energy transfer is 50%) and r is the donor-to-acceptor (D-A) distance.

Time-resolved fluorescence resonance energy transfer (TR-FRET) combines the advantages of time-resolved fluorescence and FRET. Time resolution is used to separate the energy transfer signal from the acceptor prompt fluorescence and background. (Figure 3)

The purpose of this paper is to describe the optimization process of measurement parameters using the scanning features of Thermo's Varioskan® and SkanIt® software. The quality of the assay was compared using kit default and optimized parameters and with signal-to-noise ratios and Z' values.
The assay type used for the study was a tyrosine kinase measurement. Tyrosine kinases are enzymes that can transfer a phosphate group to a tyrosine residue in a protein. They have an important role in many cellular processes therefore are of great interest in research in drug discovery.
The principle of the assay in question is to detect the relative amount of phosphorylation. It utilizes Europium labeled anti-phosphotyrosine antibody, APC streptavidin and biotinylated substrate.

Material and Methods

The optimization was made with LANCE™ Tyrosine Kinase startup reagents (PerkinElmer, product number AD0121). The assay procedure was made according to the kit insert. Eight replicates of both signal (energy transfer reaction) and noise (no energy transfer) samples were pipetted to white 384 plate (Thermo Electron, No 8155).
The measurement parameters for Varioskan were determined from the spectra and decay curve. These values were compared with the default values gained from the reagent package information.
The optimization was started with the determination of the excitation and emission wavelengths. The significant values for this assay are donor excitation and acceptor emission, therefore these were the wavelengths concentrated on this study.
The next step was to define the optimal delay time for this specific assay. This was made using the decay calculation feature of SkanIt software.
The sample plate was measured with different parameter sets and signal-to-noise and Z' values were calculated separately for each of the measurements.

Table 1. Measurement parameter sets tested

	Reference	Test 1	Test 2	Test 3	Test 4
Donor excitation (nm)	340	330	330	330	330
Acceptor emission (nm)	665	665	665	665	665
Delay	50 µs	40 µs	60 µs	70 µs	80 µs
Integration time (µs)	1000				
Measurement time (ms)	1000				

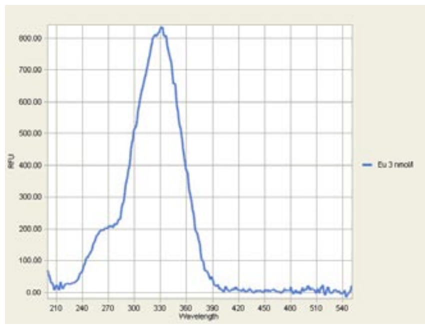

Figure 4. Europium excitation spectra. Based on this measurement the excitation wavelength 330 nm was chosen.

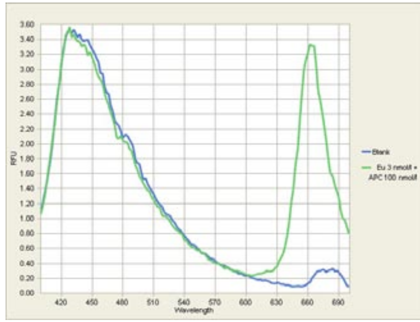

Figure 5. Energy transfer emission spectra of sample and blank wells.

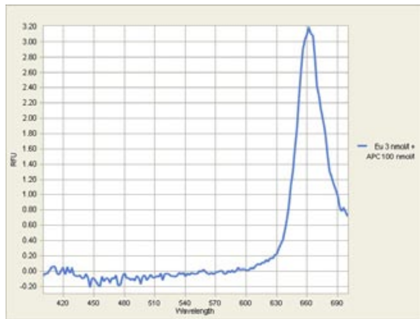

Figure 6. Emission spectra after blank subtraction. Based on this, the emission wavelgnht 665 was used.

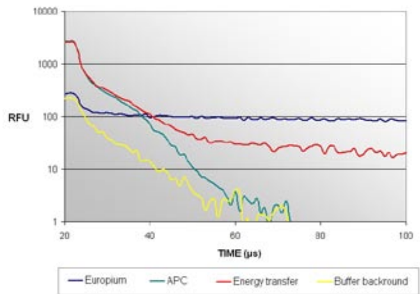

Figure 7. Decay curves for Europium, APC, APC energy transfer and assay background

Results

The emission spectra of donor and donor-acceptor pair were both run at the beginning of the optimization process. (Figure 4-6)

Based on these measurements it was decided to choose 330 nm as excitation wavelength and 665 nm emission wavelength for the energy transfer signal.
The following step for the optimization was to choose the delay time. This was made by running the decay curves for Europium, energy transfer and for background. (Figure 7)

Based on this data 50-60 µs seems to be the value, where most of the background has decayed and the sensitized emission is still reasonably high. In addition to this value also other values (40, 70 and 80 µs) were used as reference values.

The signal to noise ratio of the assay is decreased, but the Z' value of the assay is slightly improved when delay 60 is used. However, the differences are minor as it should be in the case of a commercial, well known assay and it could be successfully run with Varioskan using any of the parameter sets tested. (Table 2)

Conclusions

Varioskan® can be easily used for both TR-FRET assay measurement and assay optimization. Because of the possibility to visually examine the results, the optimization process is informative.
In addition to the graphical data used here, the delay calculation also reports numerical values for α ,τ and the recommended integration time which may be very useful for assay development.
The spectral data can be processed easily; e.g. the blank spectra reduced from the sample data. This makes the analysis of the matrix and its possible effect easier, which is important for a homogeneous assay type.
Optimization has an effect on the assay quality. The differences within the results are minor in this type of commercial assay, but at the beginning of assay development, the benefit gained should be significant.

References

1. Förster, F. Ann. Physik 1948:2:55.

2. Principles of Fluorescence Spectroscopy, Lakowicz, J.R. Plenum Publishers, New York (1999)

3. Application note: How to optimize a Tyrosine Kinase Assay Using Time Resolved Fluorescence Based LANCETM Detection, PerkinElmer

Table 2. The test values were compared with the reagent manufacturer default values. The results of the comparison runs are a mean of three different runs.

	Reference	Optimized 40 µs	Optimized 60 µs	Optimized 70 µs	Optimized 80 µs
Signal	114,0	135,3	117,4	106,7	104,4
Noise	13,3	17	14,8	14,2	13,5
SDSignal	3,8	5,5	3,4	3,8	3,7
SDNoise	2,7	2,7	2,5	2,4	2,0
S/N	8,6	8,0	7,9	7,5	7,7
Z'	0,81	0,79	0,83	0,80	0,81

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