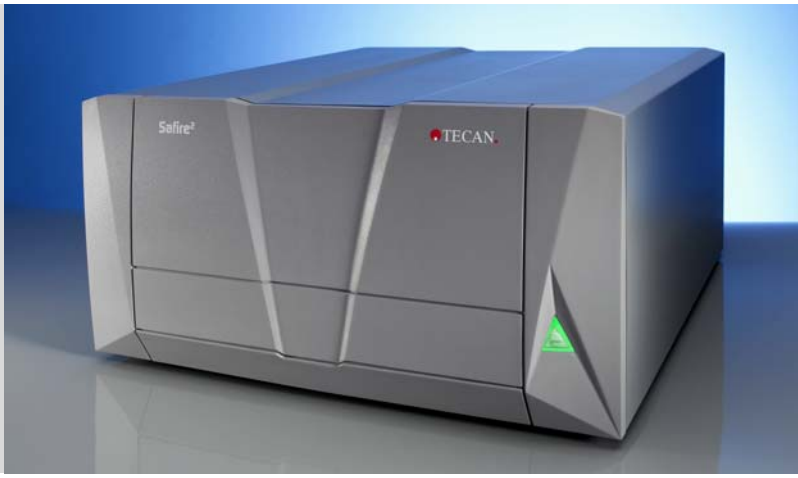
Implementation of HTRF® on Tecan Safire²™

cAMP HiRange kit, Cisbio international



Abstract

This technical note describes the implementation of HTRF® (homogeneous time-resolved fluorescence) (Cisbio international, France) measurements on Tecan's Safire²™ multifunctional and dual monochromator-based microplate reader. The cAMP (cyclic adenosine 3',5'-monophosphate) HiRange kit, which is based on HTRF® technology, was used to validate the Safire²™.

Introduction

HTRF® technology is based on the energy transfer between two fluorescent labels, a long-lifetime europium (Eu^{3+}) cryptate donor and either XL665 (chemically modified allophycocyanin) or the new d2 acceptor (1, 2). This technique combines both time-gated fluorescence (commonly referred to as time-resolved fluorescence) and fluorescence resonance energy transfer (FRET).

In recent years, time-gated fluorescence techniques have become well suited and popular for many pharmacological applications. The main benefit of time-gated measurements is the efficient reduction of background fluorescence by temporal discrimination. In addition, the energy transfer mechanism further minimizes undesirable assay interferences and side effects (e.g. volume/meniscus, quenching, light scattering, autofluorescence, molecular size) that exist in other fluorescence techniques like fluorescence intensity, fluorescence polarization and others (3).

Assay description

The cAMP HiRange kit is used for the direct quantitation of native cAMP levels produced by cells (4). It is based on the principle of HTRF® technology, where assays yield a distance-related signal. When the two labels, Eu^{3+} cryptate and XL665 or d2, are in close proximity, the signal generated by energy transfer is high. The proximity is mediated by the actual biochemical system examined.

In the present competitive immunoassay, Eu^{3+} cryptate is conjugated to anti-cAMP antibody (Ab), while the dye d2 is conjugated to cAMP. This d2-labelled cAMP can be competitively dissociated from the bound antibody by high concentrations of native cAMP. At low concentrations of unlabelled cAMP, the pre-bound complexes (Ab-cryptate and cAMP-d2) remain intact and the energy transfer is high. Increasing concentrations of competitor replace the labelled

cAMP on the antibody, leading to a decrease in energy transfer. Therefore, maximum energy transfer occurs when samples do not contain cAMP.

Material and methods

Instrument

Tecan Safire²™ monochromator-based microplate reader

Microplates

Experiments were performed in white 384-well flat bottom plates (Greiner® Cat. No. 781080). The corresponding plate definition file (GRE384fw.pdf) was selected from the available list of Tecan's XFluor4™SafireII software (Version V4.62n).

NOTE: Only *WHITE* microplates should be used for HTRF® measurements on the Safire²™

Reagents

The cAMP HiRange kit was kindly provided by Cisbio international (France), and cAMP standards were diluted according to the kit instructions (Cat. No. 62AM6PEB). Each point of the cAMP standard curve was prepared in 12 replicates with assay volumes of 80 µl. Initial cAMP concentrations are listed in table 1. Reagents were dispensed in the following order: 40 µl of cAMP standard in diluent, 20 µl cAMP-d2, and 20 µl anti cAMP-cryptate, both in reconstitution buffer. In negative controls the standard was replaced by the same volume of appropriate diluent, and cAMP-d2 by reconstitution buffer so that there was no energy transfer. The plates were covered and incubated for 1 hour at room temperature.

Standards	cAMP [nM]
Std8	2800
Std7	700
Std6	178
Std5	43.75
Std4	10.94
Std3	2.73
Std2	0.68
Std1	0.17
Std0 (max)	0

Table 1: Dilution series of cAMP standards

Measurement

HTRF® measurements were set up using the 'multilabelling' function of Tecan XFluor4™SafireII software. The Eu^{3+} cryptate (donor) was excited at 317 nm (bandwidth 20 nm). The cryptate and d2 (acceptor) emissions were detected at 620 nm (BW 10 nm) and 665 nm (BW 10 nm), respectively, using the measurement parameters listed in table 2. Number of reads (number of flashes) should be considered as recommendations (see results). The correlation between measurement time and data quality (expressed as z' values) can be found in table 4.

Measurement 1	
Ex wavelength	317 nm
Ex bandwidth	20 nm
Em wavelength	620 nm
Em bandwidth	10 nm
Lag time	60 µs
Integration time	500 µs
Number of reads	50*
Optimal gain	
Manual z-position**	

Measurement 2	
Ex wavelength	317 nm
Ex bandwidth	20 nm
Em wavelength	665 nm
Em bandwidth	10 nm
Lag time	60 µs
Integration time	500 µs
Number of reads	50*
Optimal gain	
Manual z-position**	

Table 2: HTRF® measurement parameters on Tecan Safire²™ using the 'multilabelling' option of Tecan XFluor4™SafireII software

* compare table 4
**same z-position was used for measurement 1 and 2

Calculation

The energy transfer is calculated as follows:

$$\text{Ratio} = \frac{A_{665}}{D_{620}} * 10000$$

A_{665} = Emission at 665 nm [RFU]
D_{620} = Emission at 620 nm [RFU]

$$\text{Mean Ratio} = \frac{\sum \text{ratios}}{\text{No. of replicates}}$$

$$CV = \frac{SD}{\text{Mean ratio}} * 100$$

CV = Coefficient of variation
SD = Standard deviation

$$\text{Delta F} = \frac{\text{Calibrator or sample Ratio} - \text{Ratio}_{neg}}{\text{Ratio}_{neg}} * 100$$

$Ratio_{neg}$ = Ratio of negative control

The fluorescence ratio is a correction method developed by Cisbio international, and is limited to the use of HTRF® reagents and technology. The method is covered by US patent 5,527,684 and its foreign equivalents.

Results and discussion

Table 3 represents an example of data reduction and demonstrates how the various values such as Ratio and Delta F (DF) are derived from raw data (RFU values). Sequential measurements of the donor (D_{620nm}) and the acceptor (A_{665nm}) signals yield twelve replicate values of each per sample concentration. While the donor intensities remain constant across the entire dilution series, the acceptor intensities decrease with increasing cAMP concentration, indicating the loss of energy transfer. For each individual sample the ratio of the two intensities is calculated and, subsequently, the mean ratio of the twelve replicates is calculated. From these values the relative energy transfer rate is determined as DF. DF represents the percentage increase of the FRET signal relative to the negative control.

cAMP Std [nM]	A_{665nm} [RFU*]	D_{620nm} [RFU*]	Mean Ratio	CV [%]	DF [%]
Negative control	2351	38272	614	11	
0	46280	30373	15279	8	2386
0.17	49134	32544	15104	9	2358
0.68	49003	32219	15248	5	2381
2.73	44488	32576	13681	4	2126
10.94	35697	34054	10498	4	1608
43.75	21835	36770	5939	6	866
175	10340	37616	2746	6	347
700	4714	38096	1239	6	102
2800	2783	39610	704	11	15

Table 3: Example of raw data and data reduction of one representative cAMP standard curve measured on Tecan Safire²™ *RFU values shown represent the average of 12 replicates

The variation in the DF, in relation to cAMP concentration, is shown in figure 1, displaying the cAMP standard curve measured on the Safire²™ after 1 hour of incubation. On the semi-logarithmic scale, the sigmoid curve shape follows the competitive binding event, as expected. The z' value was calculated from the minimum and maximum value in figure 1 and, a z' value of 0.74 obtained for the assay shown, is excellent. The EC50 value of 24 nM is also comparable to expected values (4).

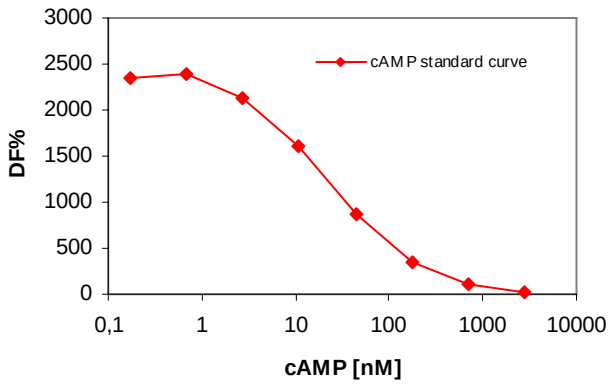


Figure 1: HTRF® cAMP calibration curve measured on the Safire²™ after 1 h of incubation using white 384-well plates (Greiner®). EC50 = 24 nM; z' = 0.74

In any assay situation the quality of the readout is questioned. The z' value (5) was developed in order to have a simple way of assessing the suitability of data. Table 4 shows the correlation between z' value versus measurement time. The z' values in table 4 represent the average data obtained from cAMP HiRange assays performed on four different Safire²™ instruments. Based on the results of table 4, we recommend using 50 flashes for HTRF® measurement on the Safire²™. Although 100 flashes would further increase the z' value, in our opinion, the resulting marginal improvement in data quality would not justify the increase in measurement time.

Safire²	**Number of flashes**			
	10	**30**	**50**	**100**
measurement time (min)	4:10	10:50	17:30	≈30:00
z' value	0.784	0.862	0.882	0.901

Table 4: Measurement time vs z' value. The z' values (calculated between the minimum and maximum of the standard curve) represent the average data from four Safire²™ instruments using the cAMP HiRange assay. Measurement times correspond to the reading time of an HTRF® assay in a 384-well plate including plate in/out but no gain or z optimization.

Conclusion

This technical note describes the successful implementation of HTRF® measurements on the Tecan Safire²™ multifunctional and dual monochromator-based microplate reader. The Safire²™ was demonstrated to be a well-suited detection platform regarding sensitivity and dynamic range of the cAMP HiRange assay kit, the HTRF® application investigated here.

Acknowledgement

The reagents for this technical note were kindly provided by Cisbio international (France). We would like to thank Fabienne Chevallier, Alexandre Jean and Francois Degorce (Cisbio international) for their constant help and valuable comments.

April, 2006

Literature

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(3) Fluorescence Lifetime (FLT): A comparative report which demonstrates FLT efficacy in HTS relative to other detection/assay technologies. Application Note. www.tecan.com

(4) cAMP HiRange: Package insert (Cisbio international, France)

(5) Zhang *et al.*, J. Biol. Screening, 4, 67 (1999)

List of abbreviations

A	Acceptor
Ab	Antibody
BW	Bandwidth
cAMP	cyclic adenosine 3',5'-monophosphate
CV	Coefficient of variation
D	Donor
DF	Delta F
EC50	Median Effective Concentration
Em	Emission
Eu	Europium
Ex	Excitation
FRET	Fluorescence resonance energy transfer
HTRF®	Homogenous time-resolved fluorescence
HTS	High throughput screening
RFU	Relative fluorescence unit
SD	Standard deviation

394 464 V1.0, 05-2006

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