Comparison of HTRF® compatible microplate readers from Tecan

Ultra Evolution™, GENios Pro™ & Safire2™ - cAMP HiRange kit

Abstract

This technical note describes the comparison of HTRF® (homogeneous time-resolved fluorescence) (Cisbio international, France) measurements on the Ultra Evolution™, GENios Pro™ and Safire2™, three multifunctional microplate readers from Tecan. The cAMP (cyclic adenosine 3',5'-monophosphate) HiRange kit, which is based on HTRF technology, was used for comparing the performance of the three detection systems.

Introduction

HTRF technology is based on the energy transfer between two fluorescent labels, a long-lifetime europium (Eu^{3+}) cryptate donor and either the 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-labeled cAMP can be competitively dissociated from the bound antibody by high concentrations of native cAMP. At low concentrations of unlabeled cAMP, the pre-bound complexes (Ab-cryptate and cAMP-d2) remain intact and the energy transfer is high. Increasing concentrations of competitor replace the labeled 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

Instruments

Filter-based detection systems
- Tecan Ultra Evolution
- Tecan GENios Pro

Monochromator-based detection system
- Tecan Safire²

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™ software.

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

Reagents

The cAMP HiRange kit was kindly provided by Cisbio international, and cAMP standards were diluted according to the kit instructions (Cat. No. 62AM6PEB). Initial cAMP concentrations are listed in table 1. Reagents were dispensed in a final volume of 80 µl per well in the following order: 40 µl of 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 plate was covered and incubated for one hour at room temperature. Each point of the cAMP standard curve was prepared in 12 replicates.

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 parameters

HTRF measurements were set up using the 'multilabeling' function of the Tecan XFluor4 software (table 2):

Measurement 1	UEvo	GPro	S²
Ex wavelength (nm)	320	320	317
Ex bandwidth (nm)	-	-	20
Em wavelength (nm)	620	620	620
Em bandwidth (nm)	-	-	10
Mirror	Dichroic2 (e.g. FI 96)	Dichroic3 (e.g. FI 96)	-
Lag time (µs)	150	150	60
Integration time (µs)	500	500	500
Number of reads	10	10	50
Gain	manual	manual	manual
z-position	manual	-*	manual

Measurement 2	UEvo	GPro	S²
Ex wavelength (nm)	320	320	317
Ex bandwidth (nm)	-	-	20
Em wavelength (nm)	665	665	665
Em bandwidth (nm)	-	-	10
Mirror	Dichroic2 (e.g. FI 96)	Dichroic3 (e.g. FI 96)	-
Lag time (µs)	150	150	60
Integration time (µs)	500	500	500
Number of reads	10	10	50
Gain	manual	manual	manual
z-position	manual	-*	manual

Table 2: HTRF measurement parameters on Tecan Ultra Evolution (UEvo), GENios Pro (GPro) and Safire² (S²). For Ultra Evolution and GENios Pro, the HTRF upgrade kit (# 10122175) was used, containing the optimized filter set for HTRF measurements: Excitation (Ex) 320/25 nm, Emission (Em) (donor) 620/10 nm and Em (acceptor) 665/8.5 nm.
**z-positioning is not possible on the GENios Pro.*

Read settings: The number of reads (number of flashes) on the Safire² should be considered as a recommendation. The correlation between measurement time and data quality (expressed as z' values) can be found in table 3.

Gain and z-position settings: We recommend preliminary experiments to acquire PMT gain and z-position, and set the corresponding values manually. Both PMT gain and z-position should be optimized on maximum signal wells using the 620 nm readout (measurement 1). The manually obtained values can be used for both HTRF readouts – 620 nm and 665 nm emission – and for subsequent measurements as long as assay type, assay volume and plate format remain the same. Manual gain setting then allows fluctuations in cryptate concentration to be monitored.

Results and discussion

Calculations for all measurements were performed according to the description in the appendix. The relative variation in the energy transfer signal expressed as Delta F (DF) is shown in figures 1 and 2. Both figures display the cAMP standard curve determined from twelve replicates measured on the Ultra Evolution, GENios Pro (fig. 1) and Safire² (fig. 2) after one hour of incubation. On the semi-logarithmic scale, the sigmoid curve shape follows the competitive binding event on all three instruments. The EC50 values of 23 - 24 nM on the Ultra Evolution, GENios Pro and Safire² are comparable to expected values (4).

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 3 shows the z' values obtained for the measurement times used. The z' values obtained for this assay were excellent on all three instruments. However, the monochromator-based Safire² requires 50 flashes in order to reach comparable data quality, which clearly increases measurement time.

	UEvo	GPro	S²
Number of reads	**10**	**10**	**50**
measurement time (min)	10:10*	6:25*	17:30**
z' value	0.771	0.763	0.749***

Table 3: Measurement time versus z' values on Ultra Evolution, GENios Pro and Safire². z' values were calculated between the minimum and maximum values in the standard curves.
**Measurement time of the two filter-based systems represent the reading time of a HTRF assay in a 384-well plate including plate in/out, gain- and z-optimization. As GENios Pro uses fixed-focus points, no z-positioning is possible.*
***Measurement time on Safire² represents the reading time including plate in/out but no gain-or z-optimization*
****Average of two different instruments*

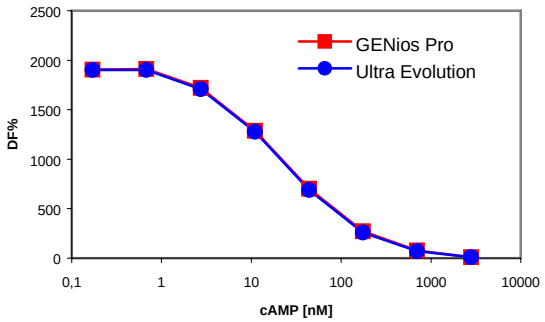

Figure 1: HTRF cAMP calibration curve measured on Ultra Evolution and GENios Pro after incubation for one hour. EC50 = 23nM

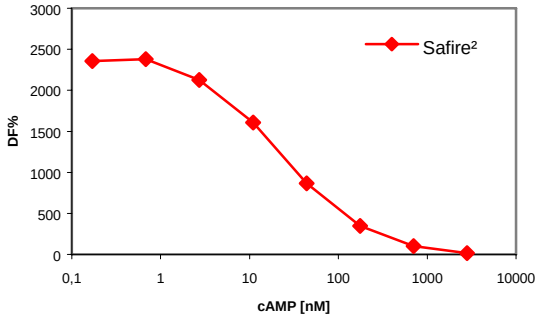

Figure 2: HTRF cAMP calibration curve measured on Safire² after incubation for one hour. EC50 = 24nM

Conclusion

HTRF assays were successfully evaluated on the two multifunctional filter-based readers, Tecan's Ultra Evolution and GENios Pro, as well as on the Tecan Safire² dual monochromator-based platform. All three instruments were demonstrated to be well-suited detection systems and the compatibility regarding sensitivity and dynamic range for HTRF assays was confirmed by Cisbio international.

Literature

(1) Modulation Processes Involved in FRET. www.htrf-assays.com

(2) The introduction of a new HTRF acceptor (d2). www.htrf-assays.com

(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
cAMP	cyclic adenosine 3',5'-monophosphate
D	Donor
DF	Delta F
Em	Emission
Eu	Europium
Ex	Excitation
FRET	Fluorescence resonance energy transfer
GPro	GENios Pro
HTRF	Homogenous time-resolved fluorescence
HTS	High throughput screening
PMT	Photomultiplier tube
RFU	Relative fluorescence unit
S²	Safire²
UEvo	Ultra Evolution

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Appendix

Calculation of Delta F*

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.

Calculation of z' value (5)*

$$z' = 1 - \frac{3 \times (SD_{max} + SD_{min})}{DF_{max} - DF_{min}}$$

SD_{max} = Standard deviation of maximum Ratio value
SD_{min} = Standard deviation of minimum Ratio value
DF_{max} = Delta F maximum value
DF_{min} = Delta F minimum value

*To simplify data reduction, Ratio, DF, EC/IC50, and z' value calcuations will be offered as a dedicated HTRF application example in Tecan Magellan™ 6.x software.

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