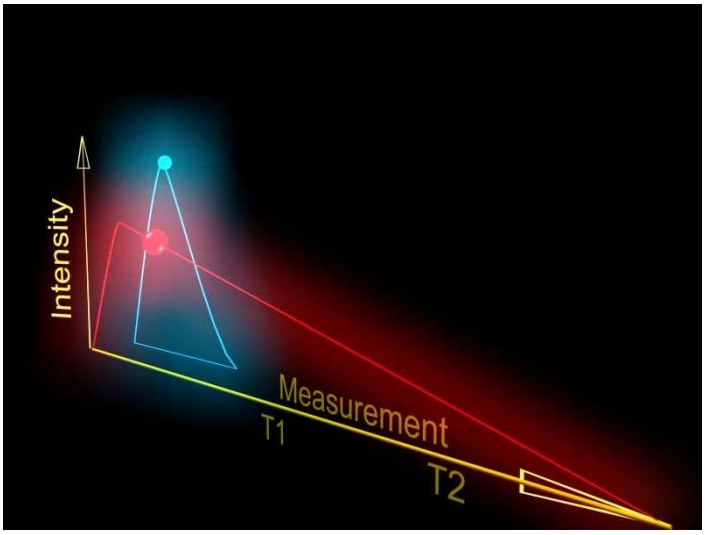
Implementation of HTRF® on Tecan GENios Pro

Human TNFα and cAMP kit, CIS bio international



Abstract

The current technical note introduces two applications implemented and verified on the GENios Pro, one of Tecan's multifunctional microplate readers. These applications utilise HTRF® (Homogeneous Time-Resolved Fluorescence) technique to generate the assay readout (CIS bio international, France).

Introduction

HTRF® (Homogeneous Time-Resolved Fluorescence) technology is based on the energy transfer between two fluorescent labels, a long-lifetime Eu^{3+}-cryptate donor and the XL665 acceptor (chemically modified allophycocyanin) (1). 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 widely used methods 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 minimises a couple of undesired assay interferences and side effects (eg. volume/meniscus, quenching, light scattering, autofluorescence, molecular size, etc.) that are present in other fluorescence techniques like Fluorescence Intensity (FI), Fluorescence Polarisation (FP), and others (2).

Especially the homogenous format of these assays, so called 'mix and measure' protocols, satisfies the demand from the industry for one-step, non-separating applications. The two main providers for time-gated homogenous assays are CIS bio international (HTRF®) and Perkin Elmer (LANCE™).

This technical note describes the implementation of HTRF® measurement using Tecan's GENios Pro plate reader.

Assay description
The human cytokine tumor necrosis factor (TNFα) and the second messenger cyclic AMP (Adenosin monophosphate) (cAMP) assay were used as examples. Both HTRF® assays yield a distance related signal. Whenever the two labels, Eu^{3+}-cryptate and XL665, are in close proximity, the signal generated by energy transfer is high. The proximity is mediated by the actual biochemical system examined.

The first assay monitors the concentration of TNFα in solution (5). Monoclonal antibodies (MAb) recognizing two distinct-epitopes are labeled with Eu^{3+}-cryptate donor and cross-linked allophycocyanin (XL665) acceptor, respectively. When TNFα is present, both MAbs bind to one such molecule, whereby they come into close proximity of each other. The higher the TNFα concentration, the more MAb pairs are formed and the higher is the energy transfer signal. As long as the tested TNFα concentration is much lower than the MAb concentration, the signal increases linearly with the TNFα concentration.

The second assay is a competitive immunoassay, which monitors cyclic AMP (cAMP) levels (3). In this assay, europium cryptate is conjugated to anti-cAMP antibody, while XL665 is conjugated to cAMP. This pre-bound XL665-cAMP can be competitively dissociated by high concentrations of free, non-labeled cAMP. At low concentrations of non-labeled cAMP the pre-bound complexes remain intact and the energy transfer is high. Increasing concentrations of competitor replace the labeled cAMP on the Ab, leading to a decrease in energy transfer. As long as the Ab concentration is kept well below the tested cAMP concentration, the generated signal will follow a sigmoidal binding curve. Maximum energy transfer occurs when samples do not contain cAMP.

From both assays, the calibration curves were considered as examples for HTRF® assay setup.

Material and Methods

Instrument:
Tecan GENios Pro

Reagents:
The Human TNFα and cAMP HTRF® reagent sets were kindly provided by CIS bio international (France).

Human TNFα HTRF®: the assay was prepared according to the TNFα kit insert (Cat. No. 62TNFPEB) (4).

HTRF® cyclic AMP dynamic kit: the calibrators were prepared according to the kit description (Cat. No. 62AM2PEB) (3).

Each point of the calibration curves was prepared in triplicate and in 100 µl assay volume per well. TNFα and cAMP concentrations are listed in Tables 1 and 2. As both assays are used in conjunction with cell cultures, the calibrators were diluted in culture medium (RPMI1640 + 10 % FCS). TNFα calibrators were diluted in both, culture medium and diluent buffer (provided with the kit). Reagents were dispensed in the following order: 50 µl of standard or sample, 25 µl cryptate, 25 µl XL665 conjugate. The negative controls were designed such that no energy transfer occured. Therefore, in negative controls the standard was replaced by the same amount of appropriate diluent. The plates were covered with a plate sealer during the incubation at room temperature. Incubation periods: cAMP 1 hour; TNFα overnight.

Table 1: TNFα calibration curve
Dilution series of TNFα calibrators performed in dilution buffer and in culture medium

Calibrator (Cal)	TNFα [pg/ml]
Cal7 (max)	2000
Cal6	1000
Cal5	500
Cal4	200
Cal3	100
Cal2	50
Cal1	20

Table 2: cAMP calibration curve
Dilution series of cAMP calibrators. Dilution was performed in culture medium (RPMI + 10 % FCS)

Calibrator (Cal)	cAMP [nM]
Cal7	712
Cal6	178
Cal5	44,5
Cal4	11,1
Cal3	2,78
Cal2	0,69
Cal1	0,17
Cal0 (max)	0

Measurement:
The measurement was set up using the 'multilabeling' function of Tecan XFluor4GeniosPro software. The Eu^{3+}-cryptate (= Donor) was excited with 320/25 nm. The cryptate emission was detected at 620/10 nm, the XL665 emission (= Acceptor) at 665/8,5 nm using the measurement parameter listed in Table 3. The filters are available as parts of the Tecan HTRF® upgrade kit (B122175; SAP No. 10122175).

Table 3: Measurement parameter
HTRF® measurement parameter on Tecan GENios Pro using the 'multilabeling' option of Tecan XFluor4GeniosPro software

Measurement 1	
Ex Filter	320 nm
Em Filter	620 nm
Mirror	Dichroic3 (eg FI 96)
Lag time	150 µs
Integration time	500 µs
Number of flashes	10
Optimal gain	

Measurement 2	
Ex Filter	320 nm
Em Filter	665 nm
Mirror	Dichroic3 (eg FI 96)
Lag time	150 µs
Integration time	500 µs
Number of flashes	10
Optimal gain	

Plate:
The following microplates were used:
- 96 Well Half Area Black Flat Bottom Plate (Corning® Cat.-No. 3686)

The corresponding plate definition file (COS96fb.pdf) was selected from the available list of Tecan's XFluor4GeniosPro software (Version V4.53).

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]

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

No. of replicates = Number of replicates

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

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 CIS bio international, which application is limited to the use of HTRF® reagents and technology. The method is covered by the US patent 5,527,684 and its foreign equivalents.

Results and Discussion

Table 4 gives an example that demonstrates how the various values (Ratio; Mean Ratio; Delta F) are derived from the raw data (RFU values). Sequential measurement of the donor (D_{620nm}) and the acceptor (A_{665nm}) signal yield to three replicate values each per sample concentration. While the donor intensities remain constant across the entire dilution series, the acceptor intensities increase with the TNFα concentration, representing the energy transfer happened. For each individual sample the ratio of the two intensities is calculated. Subsequently, the average value of the three replicates is formed and from these mean values the relative energy transfer rate is determined as Delta F (%). It measures the percentage increase of the FRET signal relative to the negative control.

The relative variation of the energy transfer signal (Delta F) is shown in Figure 1 and 2 for the two assays. Figure 1 displays two TNFα calibration curves after overnight incubation, using buffer or culture medium as respective diluents. The Delta F value increases linearly with the TNFα concentration over the entire concentration range tested. Both curves display very similar sensitivities and the lowest standard (Cal 1 = 20 pg/ml TNFα) is with a Delta F value of 13 % clearly distinguishable from the negative control. The measurement after only 3 hours of incubation already resembled these results closely (data not shown).

Figure 2 displays the cyclic AMP calibration curve after 1 hour of incubation. On the semi-logarithmic scale, the sigmoid curve shape follows the competitive binding event.

Regarding sensitivity and dynamic range of the two HTRF® assays investigated here, the Tecan GENios Pro was demonstrated to be a well-suited detection platform.

Table 4: Raw data and data reduction
Example of raw data and data reduction of one representative TNFα calibration curve measured on Tecan GENios Pro

	A_{665nm} [RFU]	D_{620nm} [RFU]	Ratio	Mean	CV [%]	DeltaF [%]
NC	4891	40568	1206	1200	6,9	
	4405	39523	1115			
	4026	31450	1280			
Cal [pg/ml]						
20	4804	34925	1376	1360	1,0	13
	5119	37956	1349			
	5380	39676	1356			
50	6418	36797	1744	1601	10,7	33
	6391	38811	1647			
	6284	44542	1411			
100	8015	42864	1870	1865	3,6	55
	7789	43369	1796			
	8324	43156	1929			
200	10870	45042	2413	2415	0,3	101
	10055	41493	2423			
	7993	33169	2410			
500	13569	31499	4308	4203	2,8	250
	15205	37292	4077			
	17077	40440	4223			
1000	26421	39401	6706	6568	2,6	447
	26690	40315	6620			
	26534	41607	6377			
2000	38715	39003	9926	10293	3,1	758
	44987	42953	10474			
	45553	43471	10479			

Figure 1: TNFα calibration curve
HTRF® human TNFα calibration curve measured after overnight incubation on Tecan GENios Pro. Dilution was performed in buffer and in culture medium.

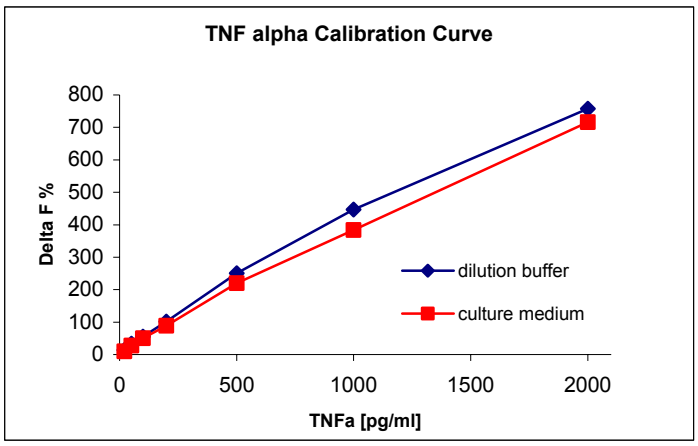
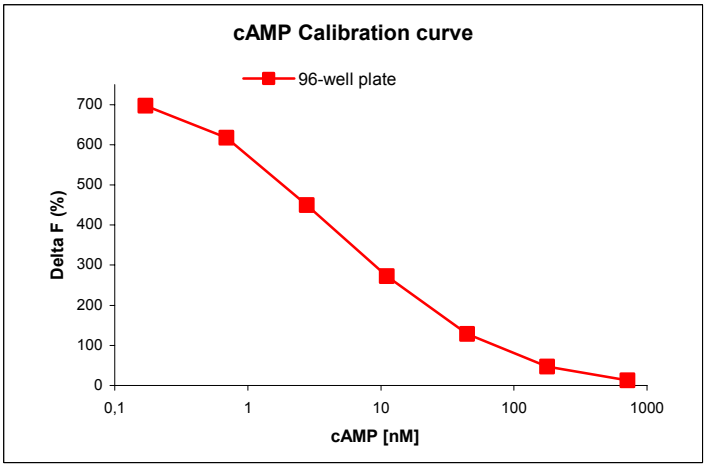


Figure 2: Cyclic AMP calibration curve
HTRF® cyclic AMP calibration curve measured after 1 h incubation using 96-well ½ area plate (Costar / Corning®).



Conclusion

HTRF® assays were successfully evaluated on the Tecan GENios Pro, a multifunctional injector reader, supporting a range of cell based assays. The data represent the excellent performance of this instrument providing a verified solution for assay development and research application.

Literature

(1) Modulation Processes Involved in FRET. http://www.htrf-assays.com/techno/fret.htm

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

(3) F. Bonnet et al.: HTRF® cyclic AMP assay: new optimized cell-based solutions for GPCR screening. http://www.htrf-assays.com

4) Human TNFα HTRF® reagent set: Package insert (CIS bio international, France)

(5) F. Degorce et al.: Assessment of human Tumor Necrosis Factor alpha (TNFα) in cell culture with a new HTRF® assay. Application Note 3. http://www.htrf-assays.com

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Glossary

A	Acceptor
cAMP	cyclic Adenosin monophosphate
CV	Coefficient of variation
D	Donor
Em	Emission
Eu	Europium
Ex	Excitation
FCS	Fetal calf serum
FI	Fluorescence intensity
FP	Fluorescence polarization
FRET	Fluorescence resonance energy transfer
HTRF®	Homogenous time resolved fluorescence
HTS	High throughput screening
MAb	Monoclonal antibody
RFU	Relative fluorescence unit
RPMI1640	Roswell Park Memorial Institute 1640 Media
TNFα	Tumor necrosis factor alpha

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