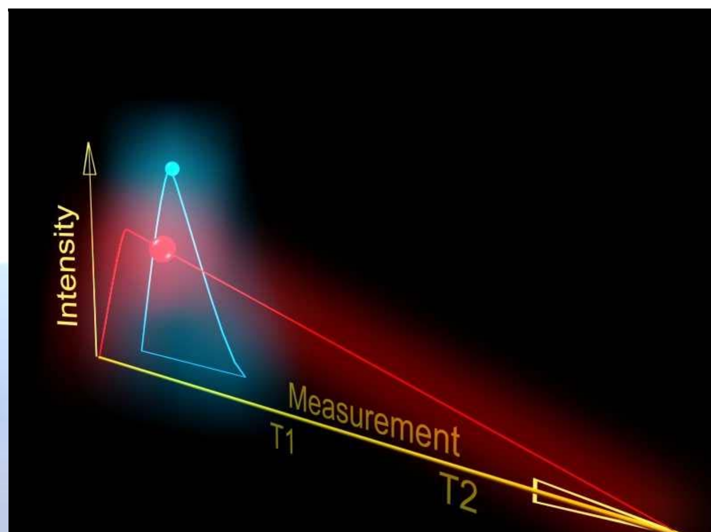
TECAN.

Implementation of HTRF® on Tecan Ultra Evolution

Human TNFα and cAMP kit, CIS bio international



Abstract

The current technical note introduces two applications, implemented and validated on the ULTRA Evolution, Tecan's multifunctional microplate reader. 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. The addition of energy transfer 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), etc. (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 for high throughput screening (HTS). The two main providers for time-gated homogenous assays are CIS bio international (HTRF®) and Perkin Elmer (LANCE™).

TECAN.

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

Assay description
Two calibration curves were considered as examples: 1) the human cytokine tumor necrosis factor (TNFα) and 2) the second messenger cyclic AMP (Adenosin monophosphate) (cAMP). These two HTRF® assays both 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). Two distinct-epitope monoclonal antibodies (MAb) 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.

Material and Methods

Instrument:
Tecan ULTRA Evolution

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, in either 100 µl or 20 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

Plate:
The following microplates were used:

- 96 Well Half Area Black Flat Bottom Plate (Corning® Cat.-No. 3686)
- 384 Well Small Volume™ HiBase Microplate; black (Greiner Bio-One, Germany) (Cat.-No. 784 076)

The corresponding plate definition files (COS96fb.pdf and GRE384sb.pdf) were selected from the available list of Tecan's XFluor4 software (Version V4.50).

Measurement:
The measurement was set up using the 'multilabeling' function of Tecan XFluor4 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).

Table 3: Measurement parameter
HTRF® measurement parameter on Tecan ULTRA Evolution using the 'multilabeling' option of Tecan XFluor4 software

Measurement 1	
Ex Filter	320 nm
Em Filter	620 nm
Mirror	Dichroic2 (eg FI 96)
Lag time	150 µs
Integration time	500 µs
Number of flashes	10
Optimal gain	
Optimal z-pos	

Measurement 2	
Ex Filter	320 nm
Em Filter	665 nm
Mirror	Dichroic2 (eg FI 96)
Lag time	150 µs
Integration time	500 µs
Number of flashes	10
Optimal gain	

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

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

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 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 may demonstrate 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 9 % 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 using two different plate types. On the semi-logarithmic scale, the sigmoid curve shape follows the competitive binding event. Both curves display the same shape, independent of the assay volume of 100 µl (96-well ½ area) or 20 µl (384-well small volume) used. The data quality is still excellent. Therefore, miniaturisation of these assays is possible with Tecan ULTRA Evolution.

Regarding sensitivity and dynamic range of the two HTRF® assays investigated here, the Tecan ULTRA Evolution was demonstrated as a suitable 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 ULTRA Evolution

	A_{665nm} [RFU]	D_{620nm} [RFU]	Ratio	Mean	CV [%]	DeltaF [%]
NC	4455	47180	944	929	6,7	
	4093	47567	860			
	3924	39928	983			
Cal [pg/ml]						
20	4982	48566	1026	1013	1,2	9
	5004	49457	1012			
	4857	48540	1001			
50	6040	46832	1290	1232	4,2	33
	5568	46878	1188			
	5867	48120	1219			
100	7649	48519	1576	1626	6,9	75
	7431	48069	1546			
	8258	47063	1755			
200	11269	49180	2291	2204	4,8	137
	10078	48270	2088			
	9397	42100	2232			
500	16131	46088	3500	3695	5,2	298
	17613	47587	3701			
	18577	47841	3883			
1000	27717	46424	5970	3695	1,0	543
	28230	46745	6039			
	27507	46490	5917			
2000	42138	46435	9075	9323	4,2	903
	42366	46464	9118			
	44266	45276	9777			

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

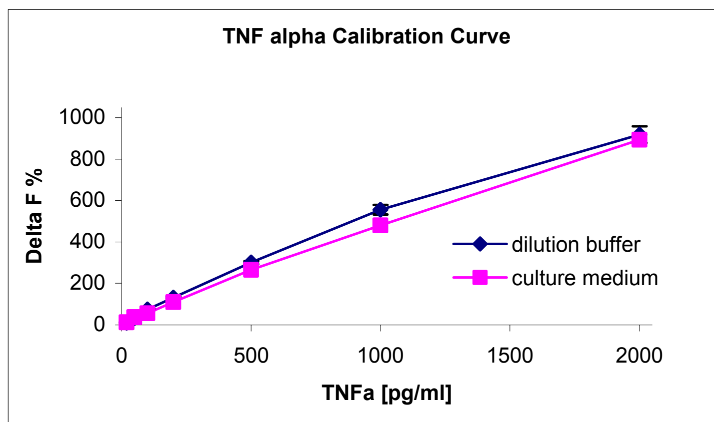
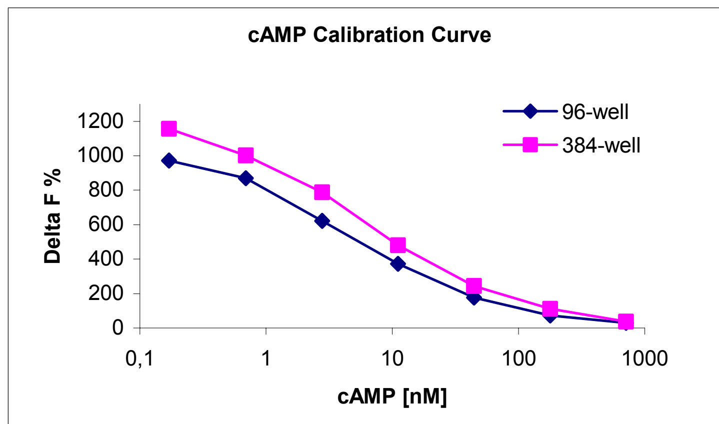


Figure 2: cyclic AMP calibration curve
HTRF® cyclic AMP calibration curve measured after 1 h incubation using 96-well ½ area and 384-well small volume plates.



Conclusion

The ULTRA Evolution, one of the multifunctional plate readers of Tecan, is compatible to HTRF® measurements. The presented homogenous assays are well suited for miniaturization and to high-throughput applications.

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

Tecan Asia (Pte) Ltd., 80, Marine Parade, #13-04, Singapore 449269, Singapore, T +65 644 41 886, F +65 644 41 836
Tecan Sales Austria GmbH, Untersbergstrasse 1a, A-5082 Grödig / Salzburg, Austria, T +43 62 46 89 33, F +43 62 46 72 770
Tecan Sales International GmbH, Untersbergstrasse 1a, A-5082 Grödig / Salzburg, Austria, T +43 62 46 89 33, F +43 62 46 72 770
Tecan Benelux B.V.B.A., Vaartdijk 55, B-2800 Mechelen, Belgium, T +32 15 42 13 19, F +32 15 42 16 12
Tecan Benelux B.V.B.A., Industrieweg 30, NL-4283 GZ Giessen, Netherlands, T +31 18 34 48 17 4, F +31 18 34 48 06 7
Tecan Deutschland GmbH, Theodor-Storm-Straße 17, D-74564 Crailsheim, Germany, T +49 79 51 94 170, F +49 79 51 50 38
Tecan France S.A., Parc d'Activités de Pissaloup, Bâtiment Hermes II, Rue Edouard Branly, F-78190 Trappes, France, T +33 1 30 68 81 50, F +33 1 30 68 98 13
Tecan Italia S.r.l., Via F.lli Cervi, Palazzo Bernini, Centro Direzionale Milano 2, I-20090 Segrate (Mi), Italy, T +39 02 215 21 28, F +39 02 215 97 441
Tecan Japan Co. Ltd, Meiji Seimei Fuchu Building 10F, 1-40 Miyamachi, Fuchu City, Tokyo, Japan, T +81 42 334 88 55, F +81 42 334 04 01
Tecan Nordic AB, Box 208, SE-431 23 Mölndal, Sweden, T +46 31 75 44 000, F +46 31 75 44 010
Tecan Portugal, Quinta da Fonte - Edificio Pedro I, 2780-730 Paço d'Arcos, Portugal, T +351 21 000 82 16, F +351 21 000 16 75
Tecan Sales Switzerland AG, Seestrasse 103, CH-8708 Männedorf, Switzerland, T +41 1 922 89 22, F +41 1 922 89 23
Tecan Spain, Sabino de Arana, 32, E-08028 Barcelona, Spain, T +34 93 490 01 74, F +34 93 411 24 07
Tecan UK, Theale Court, 11-13 High Street, Theale, UK-Reading RG7 5AH, United Kingdom, T +44 11 89 300 300, F +44 11 89 305 671
Tecan US, P.O. Box 13953, Research Triangle Park, NC 27709, USA, T +1 919 361 5200, F +1 919 361 5201

www.tecan.com

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

Acknowledgement

All the reagents were kindly provided by CIS bio international (France). We would like to thank Dr. Marc Preaudat and Dr. Sylvie Sulocha (CIS bio international) for their constant help and valuable comments.