Application Note

HTRF® (Cisbio) Human Interleukin beta (IL1β) assay

Tecan Infinite™ F500, TR-FRET (HTRF®)

Abstract

This technical note describes the implementation of HTRF® (Homogeneous Time-Resolved Fluorescence; Cisbio International, France) measurements, in particular the HTRF®-human IL1β kit on the Infinite™ F500, a filter-based multifunctional detection system.

Introduction

HTRF® technology

The HTRF® technology combines *time resolved fluorescence* (TRF) with *fluorescence resonance energy transfer* (FRET). It 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).

Time resolved fluorescence measurement techniques have become very popular for many pharmacological applications, since they enable an efficient reduction of background fluorescence by temporal discrimination. The method of fluorescence resonance energy transfer is widely used for its ability to minimize undesired assay interferences and side effects (e.g. volume/meniscus, quenching, light scattering, autofluorescence, molecular size) that exist in other fluorescence techniques (3).

Interleukin 1 beta

Interleukin 1 (IL-1) is a peptide belonging to the class of cytokines, mainly produced by activated macrophages and monocytes. It is known as a mediator of the host inflammatory response in natural immunity. IL-1 has both, paracrine effects on cells in its proximity (e.g. the stimulation of synthesis and secretion of a variety of interleukins or the activation of T cells leading to an adaptive immune response) and endocrine effects (e.g. fever induction), as IL-1 is transported through the body via the blood system. IL-1 exists in two distinct molecular forms, IL-1α and IL-1β. Both forms are synthesised from large precursors which are cleaved by caspase-1 in order to produce the active IL-1α and IL-1β, respectively.

Assay description

The human IL-1β HRTF® kit is used for the quantitative determination of human Interleukin 1 β (4). It is based on the principle of HTRF® technology, where assays yield a distance-related signal. IL-1β is detected by a system of two mouse anti IL-1β monoclonal antibodies (mouse-anti IL-1β MAbs), one labeled with XL665, the other one with Eu^{3+} cryptate. These two antibodies recognise specific epitopes of human IL-1β and by binding to their targets the energy

transfer is triggered. The resulting signal is proportional to the concentration of IL-1β in the respective sample.

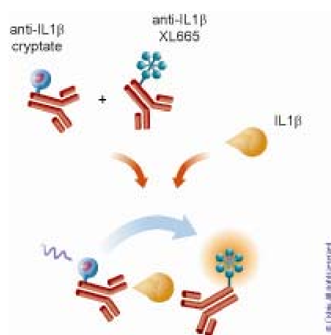

Figure 1: Assay principle for detection of human IL-1β.

Material and methods

Instrument

Tecan Infinite™ F500 filter-based microplate detection system

Microplates

384 well flat bottom white microplates (Greiner Bio-One)
384 well flat bottom black microplates (Greiner Bio-One)

Reagents and Assay Performance

The IL-1β kit was kindly provided by Cisbio International (France), and all reagents were reconstituted and diluted according to the kit instructions (4, 3.1) in either RPMI 1640 cell culture medium (PAA laboratories) or dilution buffer (provided with the kit). Table 1 shows the dilution sequence which was followed to constitute the standard curve (4, 3.3)

Standard	final conc. IL-1β [pg/ml]	Preparation
Std7	2000	1000 µl pure standard
Std6	1000	500 µl Std7 + 500 µl diluent*
Std5	500	500 µl Std6 + 500 µl diluent*
Std4	200	400 µl Stdl5 + 600 µl diluent*
Std3	100	500 µl Std4 + 500 µl diluent*
Std2	50	500 µl Std3 + 500 µl diluent*
Std1	20	400 µl Std2 + 600 µl diluent*

Table 1: Dilution sequence of IL-1β calibrator
* diluent is either RPMI cell culture medium or dilution buffer

The reagents were dispensed into micro plates in the following order: 20 µl calibrator – add 10 µl anti-IL1β-Cryptate – add 10 µl anti-IL1β-XL665. For negative control 20 µl of the dilution reagent was used instead of the standard solution (4, 3.5)

Measurements

HTRF® measurements were set up using the 'multi labelling' function of Tecan i-control software. The Eu^{3+} cryptate (donor) was excited at 320 nm (bandwidth 25 nm). The cryptate and XL665 (acceptor) emissions were detected at 620 nm (BW 10 nm) and 665 nm (BW 8 nm), respectively, using the measurement parameters listed in Table 2.

Measurement 1	
Ex wavelength [bandwidth]	320 [25] nm
Em wavelength [bandwidth]	620 [10] nm
Lag time	150 µs
Integration time	500 µs
Number of reads	10
gain	optimal
z-position	(calc. from B1)
Measurement 2	
Ex wavelength [bandwidth]	320 [25] nm
Em wavelength [bandwidth]	665 [8] nm
Lag time	150 µs
Integration time	500 µs
Number of reads	10
gain	optimal
z-position	manual - same as meas. 1

Table 2: HTRF® measurement parameters on Tecan Infinite™ F500 using the 'multi labelling' option of i-control software

Calculations

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

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

$Ratio_{neg}$ = Ratio of negative control

$$z' = 1 - \frac{3 * SD(\min .values) + 3 * SD(\max .values)}{mean(\min .values) - mean(\max .values)}$$

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. Z´ values were calculated between the minimum (20 pg/ml IL-1β) and maximum (2000 pg/ml IL-1β) values.

Results and discussion

Table 3 and 4 represent all relevant data, which were acquired by sequential measurements of the donor (D_{620nm}) and the acceptor (A_{665nm}) signals (6 replicate values per sample concentration). The donor intensities remain relatively constant across the entire dilution series, the acceptor intensities increase with increasing IL-1β concentration, indicating an enhanced energy transfer (raw data not shown). For each individual sample the ratio of the two intensities is calculated and, subsequently, the mean ratio of the six replicates is calculated. In addition the SD and the respective CV were calculated. From these values the relative energy transfer rate is determined as ΔF. Delta F represents the percentage increase of the FRET signal relative to the negative control. Figure 2 and Figure 3 show the calibration curves appropriate to the data presented in Table 3 and 4.

	GRE 384 white, culture medium					GRE 384 white, dilution buffer				
	Ratio mean	*SD*	*CV (%)*	*Δ F (%)*	*Z´*	*Ratio mean*	*SD*	*CV (%)*	*Δ F (%)*	*Z´*
IL1b					0,909					0,899
pg/ml										
NC	1941	35,8	1,8			1930	63,6	3,29		
2000	1071	239,3	2,2	451,8		1037	239,2	2,30	437,4	
1000	7	194,9	3,1	216,9		6	232,2	3,60	233,5	
500	6154	109,6	2,7	103,3		6441	66,2	1,53	123,3	
200	3948	89,5	3,2	40,6		4312	95,3	3,30	49,3	
100	2730	26,9	1,5	19,7		2883	59,6	2,49	23,5	
50	2326	26,3	1,7	10,6		2385	58,0	2,68	12,0	
20	2149	24,3	1,2	4,2		2163	39,6	1,92	6,4	
	2023					2055				

Table 3: Data summary using Greiner Bio-One 384 well white microplates and culture medium or dilution buffer as diluent.

	GRE 384 black, culture medium					GRE 384 black, dilution buffer				
	Ratio mean	*SD*	*CV (%)*	*Δ F (%)*	*Z´*	*Ratio mean*	*SD*	*CV (%)*	*Δ F (%)*	*Z´*
IL1b					0,829					0,909
pg/ml										
Neg. co	2358	124,4	5,27			2597	284,2	10,94		
2000	10842	349,0	3,21	359,8		11307	196,0	1,73	379,5	
1000	6355	288,3	4,53	169,5		7086	334,4	4,71	200,5	
500	4835	104,7	2,16	105,0		4723	215,2	4,55	100,3	
200	3603	445,8	12,37	52,8		3421	264,8	7,74	45,1	
100	3123	143,7	4,60	32,4		2979	181,5	6,09	26,3	
50	2730	301,8	11,05	15,8		2860	273,7	9,56	21,3	
20	2578	121,7	4,72	9,3		2781	60,2	2,16	17,9	

Table 3: Data summary using Greiner Bio-One 384 well black microplates and culture medium or dilution buffer as diluent.

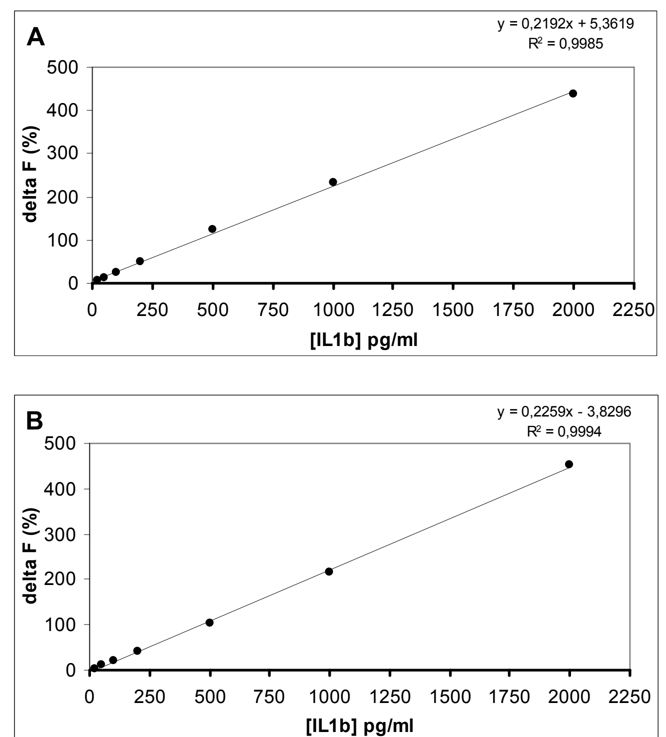

Figure 2: HTRF® IL1-β standard curve measured 2 h of incubation using *white 384-well plates* (Greiner®)
A: dilution buffer as diluent; B: cell culture medium as diluent

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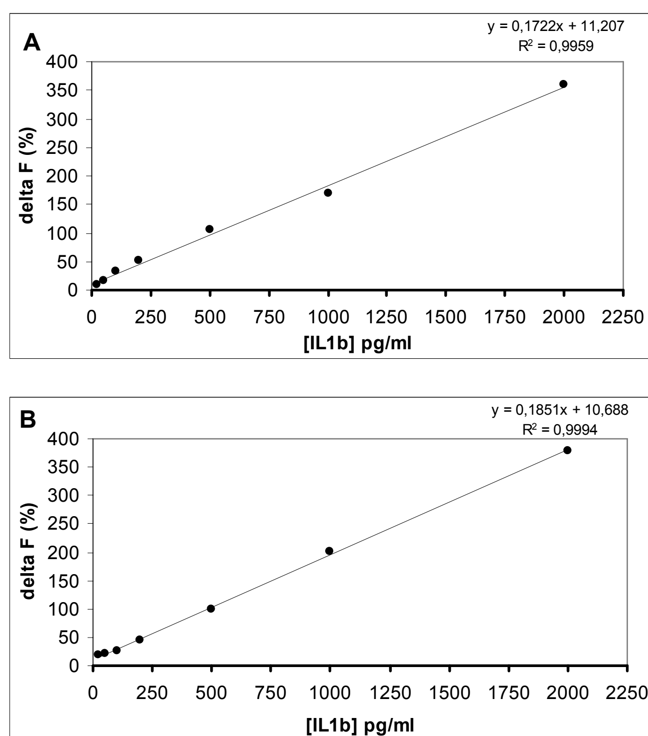

Figure 3: HTRF® IL1-β standard curve measured after 2 h of incubation using *black 384-well plate*s (Greiner®)
A: dilution buffer as diluent; B: cell culture medium as diluent

The data presented above clearly demonstrate that the Tecan Infinite™ F500 is perfectly fitting to the requirements for HTRF® measurements. All obtained z´values were excellent, even those calculated from measurements in black microplates. The dynamic ranges of the different measurements were excellent as well and the smallest concentration (20 pg/ml) of IL-1β was above the negative control level in all cases.

A final experiment was performed to check the influence of the number of flashes (reads) per well on the outcome of HTRF® measurements. The derived data are presented in Table 5. It can be proved that even with one flash per well a very good z´ value of about 0.79 can be reached and can be further improved to about 0.9 using 10 flashes.

Infinite F 500	Number of flashes		
	1	3	10
z' value	0.788	0.849	0.899

Table 5: Number of flashes (reads) per well vs. z' value using a white micro plate and dilution buffer as diluent.

Conclusion

This technical note describes the successful implementation of HTRF® measurements on the Tecan Infinite™ F500 filter based multifunctional reader. The Infinite™ F500 was proven to be a very well-suited detection platform regarding sensitivity and dynamic range of the investigated IL-1β detection - HTRF®assay kit.

Acknowledgements

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Literature

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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
IL-1β	Interleukin 1 β
HTS	High throughput screening
RFU	Relative fluorescence unit
SD	Standard deviation

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