Implementation of HTRF® Assay Technology on Infinite® F200 PRO

Reader Control Kit, cAMP HiRange Kit, TNF-alpha Kit

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Abstract

HTRF-based applications have become increasingly popular for the analysis of molecular interactions and binding studies because of their homogeneity, robustness, sensitivity, and potential for miniaturization. The technology is well established as an HTS screening tool, but also proofed to be beneficial for applications at a moderate throughput. Although the maximum speed of the HTRF reader may not be the primary requirement for the life science community, the instrument still needs to offer excellent optical properties to deliver high-quality results often associated with more expensive technical solutions.

With the Infinite F200 PRO, Tecan offers a cost-efficient yet highly sensitive and robust detection system for HTRF users at a medium throughput. The instrument is equipped with a sophisticated and ingenious optical system with a dichroic mirror, which permits optimal excitation in the UV range. Optimized filters for HTRF guarantee specific signal detection with high signal-to-noise, HTRF ratios, and Delta F values.

In this poster we describe the performance of the Infinite F200 PRO for the HTRF reader control kit, the HTRF cAMP assay and the HTRF TNF-alpha Kit. For all three assay-formats the instrument delivered the best performance in regard to signal to noise, detection limit and dynamic range of HTRF-based signals.

Introduction

HTRF combines time-resolved fluorescence (TRF) and fluorescence resonance energy transfer (FRET). It is based on the energy transfer between two fluorophores, a long-lived europium or terbium cryptate donor and either a chemically modified allophycocyanine (XL665) or a d2 acceptor. HTRF-based applications have become increasingly popular for the analysis of various molecular interactions and binding studies because of their homogeneity, robustness, sensitivity, and potential for miniaturization.

Tecan's Infinite F200 PRO is equipped with a sophisticated and ingenious mirror system containing a dichroic mirror with a break point at 510nm, which permits optimal excitation in the UV range and thus enables HTRF measurements in the Infinite F200 PRO.

This technical solution enables for high-quality results but also results in a low product price.

HTRF Reader Control Kit

The HTRF Reader Control Kit (RCK) is intended for the calibration of HTRF compatible instruments and for validation of their ability to perform HTRF measurements. The RCK contains the complete analytical chemistry that is necessary to assess the HTRF performance of the reader to be tested and can be used to compare different instruments.

cAMP HiRange Kit

Cyclic AMP (cyclic adenosine 3', 5'-monophosphate) is one of the most important intracellular messengers and is involved in numerous biological processes such as GPCRs, protein kinase activation or ion channel regulation. The cAMP HiRange kit is designed for the direct quantification of cAMP in suspension or adherent cells by means of a competitive immunoassay on the basis of HTRF technology. It permits the measurement of agonist and antagonist effects on GPCRs in different cell lines. The measured TR-FRET signal is inversely proportional to the cAMP concentration in the sample.

TNF-alpha Kit

Tumor necrosis factor (TNF)-alpha is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase immune reaction. Under inflammatory conditions TNF-alpha is secreted by activated macrophages and monocytes. The Human TNF-alpha kit allows for the quantitation of TNF-alpha in cells or supernatants by antibody-mediated recognition of two distinct epitopes that are tagged with HTRF donor and acceptor fluorophores. The HTRF ratio increases linearly with the TNF-alpha concentration.

Results

HTRF Reader Control Kit

The measurement results obtained with the Reader Control Kit demonstrate the applicability of the Infinite F200 PRO for HTRF analyses. The Infinite F200 PRO meets all performance criteria specified by Cisbio (tables 1 and 3), resulting in an exceptionally good measurement range (Delta F low = 32, Delta F high = 1189, Z' value = 0.95) and minimal measurement variations (CVs of all calibrators and standards ≤ 2%).

Reader Control Kit – Measurement results

Signal-to-Blank ratio	268
CV Standard	0 1.24 %
CV Low Calibrator	1.26 %
CV High Calibrator	1.35 %
Delta R low	278
Delta R high	10454
Delta F low	32
Delta F high	1189
Z' value	0.95

Table 3: RCK measurement results

cAMP HiRange Kit

As shown in figure 4. the Delta F values obtained with the cAMP dilution series are inversely proportional to the cAMP concentration, resulting in the sigmoidal shape of the curve that is typical for competitive assays.

cAMP HiRange Kit – Measurement results

CV Standard 0	3.44 %
Delta F Standard 8	20
Delta F Standard 7	94
Delta F Standard 6	330
Delta F Standard 5	827
Delta F Standard 4	1514
Delta F Standard 3	2130
Delta F Standard 2	2480
Delta F Standard 1	2689
Delta F Standard 0	2794
Assay window	143

Table 4: cAMP measurement results

Human TNF-alpha Kit

The measurement of a dilution series of the TNF-alpha standard shows a nearly linear course (R^2 = 0.9998) from 2000 to 20 pg/ml TNF-alpha (figure 5).

According to the evaluation protocol provided by Cisbio, only calibrators 1 to 3 were taken as a basis for the calculation of the TNF-alpha detection limit. The resulting detection limit for TNF-alpha was calculated to be 5.56 pg/ml. The detailed results (average of three independent measurements) are summarized in table 5.

Human TNF-alpha – Measurement results

CV Standard 0	1.26%
Delta F Calibrator 3	41
Delta F Calibrator 2	21
Delta F Calibrator 1	8
Linearity (R^2)	0.9998
Detection limit	**5.56 pg/ml**

Table 5: TNF-alpha measurement results

	96-well plate	384-well plate
1 flash	0:35	1:26
50 flashes	4:51	17:54

Table 6: HTRF measurement speed of the Infinite F200 PRO

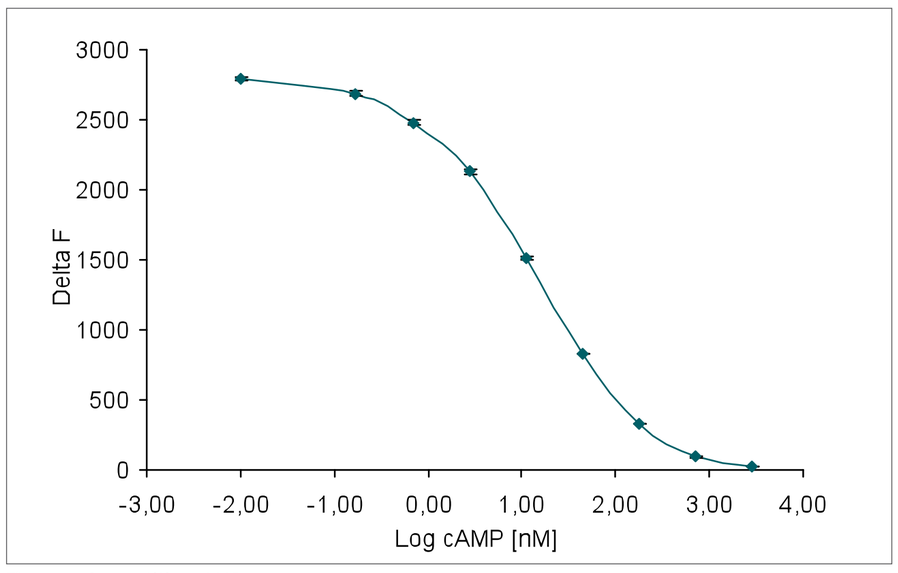

Figure 4: Competitive cAMP curve

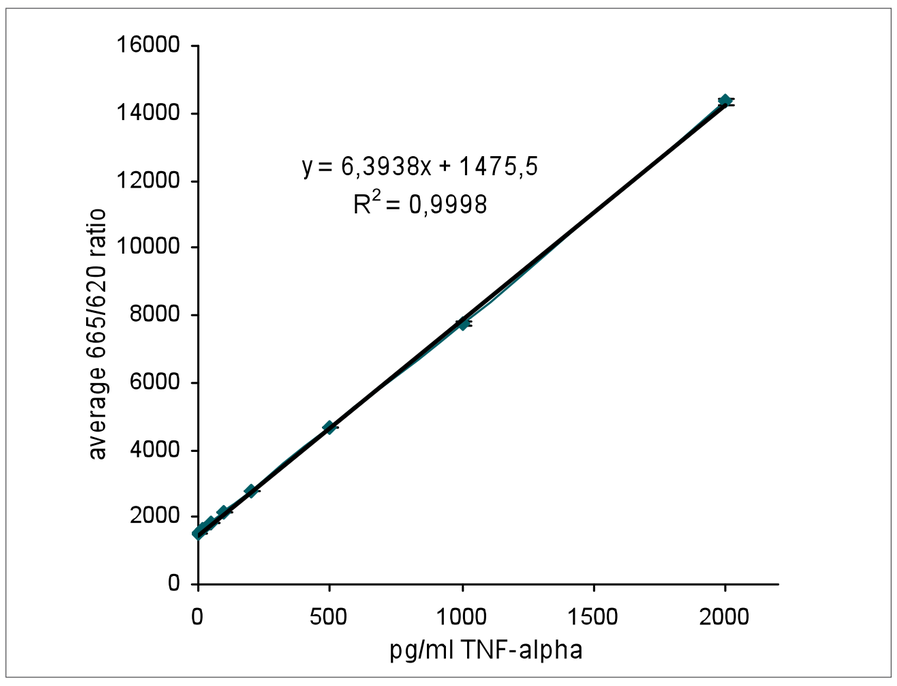

Figure 5: Linearity of TNF-alpha dilution series

Materials and Methods

Instrument

- Infinite F200 PRO filter-based multimode microplate reader

Microplates

- 96-well half area microplates, white (Greiner Bio-One, Germany)
- 384-well small volume microplates, white (Greiner Bio-One, Germany)

Reagents

- HTRF Reader Control Kit (62RCLPEA, Cisbio)
- cAMP HiRange kit (62AM6PEB, Cisbio)
- Human TNF-alpha kit (62TNFPEB, Cisbio)

Assay protocol

HTRF Reader Control Kit

The HTRF RCK was used according to the manufacturer's instructions. The measurement was performed in a white 96-well half area plate using 100 µl filling volume per well. The plate was measured in triplicates.

Figure 1: Plate layout for the HTRF Reader Control Kit
(A1-D1): 50 µl 620 nm control, 50 µl reconstitution buffer · **(A2-H3):** 50 µl diluent, 25 µl cryp, 25 µl XL665 · **(A4-H4):** 50 µl low cal. 25 µl cryp, 25 µl XL665 · **(A5-H5):** 50 µl high cal., 25 µl cryp, 25 µl XL665 · **(A6-C6):** 50 µl diluent, 50 µl reconst. buffer **(A7-C7):** 50 µl diluent, 25 µl XL665. 25 µl reconst. buffer · **(A8-C8):** 50 µl diluent, 25 µl cryp, 25 µl reconst. buffer

RCK - Passed/failed criteria

Signal-to-Blank ratio	≥ 40 = passed
CV Standard	0 ≤ 10 % = passed
CV high Standard	≤ 10 % = passed
CV low Standard	≤ 10 % = passed
Delta F low	≥ 15 = passed
Delta F high	≥ 600 = passed

Table 1: Performance criteria for HTRF certification.

cAMP HiRange Kit

The cAMP HiRange kit was used according to the manufacturer's instructions. The measurement was performed in a white 384-well small volume plate using 20 µl filling volume per well. The plate was measured in triplicates.

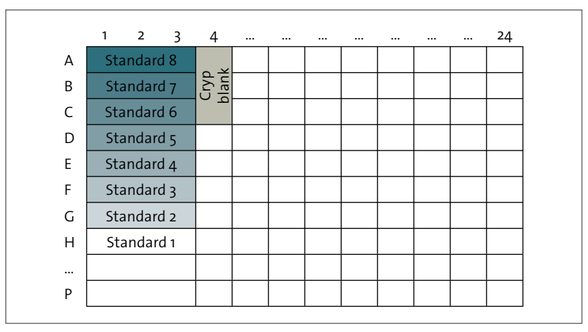

Figure 2: Plate layout for the cAMP HiRange Kit
(A1-A3): Standard 8 = 2800 nM cAMP · **(B1-B3):** Standard 7 = 700 nM cAMP **(C1-C3):** Standard 6 = 174 nM cAMP · **(D1-D3):** Standard 5 = 43.75 nM cAMP **(E1-E3):** Standard 4 = 10.94 nM cAMP · **(F1-F3):** Standard 3 = 2.73 nM cAMP **(G1-G3):** Standard 2 = 0.86 nM cAMP · **(H1-H3):** Standard 1 = 0.17 nM cAMP **(A4-C4):** Cryptate Bank = cryptate conjugate buffer

Human TNF-alpha Kit

The Human TNF-alpha kit was used according to the manufacturer's instructions. The measurement was performed in a white 384-well small volume plate using 20 µl filling volume per well. The plate was measured in triplicates.

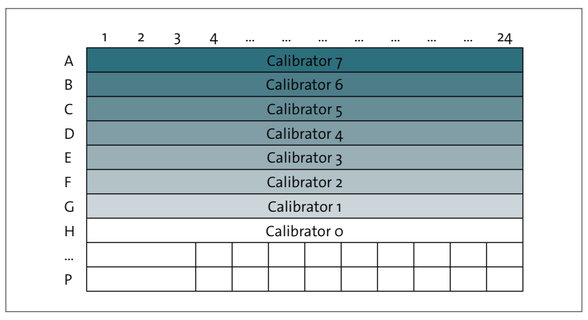

Figure 3: Plate layout for the Human TNF-alpha Kit
(A1-A24): Calibrator 7 = 2000 pg/ml TNF-alpha · **(B1-B24):** Calibrator 6 = 1000 pg/ml TNF-alpha · **(C1-C24):** Calibrator 5 = 500 pg/ml TNF-alpha · **(D1-D24):** Calibrator 4 = 200 pg/ml TNF-alpha · **(E1-E24):** Calibrator 3 = 100 pg/ml TNF-alpha · **(F1-F24):** Calibrator 2 = 50 pg/ml TNF-alpha · **(G1-G24):** Calibrator 1 = 20 pg/ml TNF-alpha **(H1-H24):** Calibrator 0 = diluent

Measurement settings

Measurement 1 (Donor)	
EX wavelength	320 (25) nm
EM wavelength	620 (10) nm
Mirror dichroic	510
Lag time	150 µs
Integration time	500 µs
Number of flashes	50
Gain	optimal
Measurement 2 (Acceptor)	
EX wavelength	320 (25) nm
EM wavelength	665 (8) nm
Mirror dichroic	510
Lag time	150 µs
Integration time	500 µs
Number of flashes	50
Gain	optimal

Table 2: Optimized instruments settings for HTRF measurements on Infinite F200 PRO

Calculations

Detection limit (TNF-alpha):
Detection limit = dose of mean zero + 2 SD

Delta R:
Delta R = Average Standard X – Average Std 0

Delta F:

$$\text{Delta F} = \frac{\text{Delta R} * 100}{\text{Average Std 0}}$$

Assay window (cAMP):

$$\text{Assay window} = \frac{\text{Delta F Std 0}}{\text{Delta F Std 8}}$$

Conclusion

In accordance with the criteria for the HTRF Reader Control Kit, the Infinite F200 PRO in combination with the 510 dichroic mirror meets all performance parameters that are relevant for HTRF-compatible readers and is thus regarded as HTRF-certified.

In addition, both cAMP and TNF-alpha can be reliably and sensitively quantified in the Infinite F200 PRO. In summary, the results of the Reader Control Kit, the cAMP HiRange Kit and the Human TNF-alpha Kit show that the Infinite F200 PRO with an integrated 510 dichroic mirror is a perfectly suited detection instrument for the measurement of various HTRF assays formats.

The great assay performance combined with reasonable measurement times and a low selling price makes the Infinite F200 PRO the instrument of choice for all customer with low- to medium throughput HTRF applications.

Abbreviations

cAMP	cyclic adenosine monophosphate
CV	coefficient of variation
FRET	fluorescence resonance energy transfer
TNF	tumor necrosis factor
TRF	time-resolved fluorescence
TR-FRET	time-resolved fluorescence resonance energy transfer
HTRF	homogenous time-resolved fluorescence
RCK	Reader Control Kit
S:B	ratio signal-to-blank ratio
SD	standard deviation

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