



Implementation of HTRF[®]

technology on the Spark[®]

multimode reader.

Application Note

**SENSITIVE, FAST AND RELIABLE MEASUREMENT OF
HIGH-THROUGHPUT SCREENING ASSAYS**



INTRODUCTION

Homogeneous Time-Resolved Fluorescence (HTRF) is a time-resolved fluorescence resonance energy transfer (TR-FRET) assay system developed by Cisbio Bioassays, and offers a reliable technique for high throughput screening (HTS) and the analysis of molecular interactions. The assay is based on energy transfer between a long-lived europium or terbium cryptate donor and a variety of red or green acceptors, and combines the advantages of FRET and time-resolved fluorescence (TRF).

HTRF assays require detection instruments with a good sensitivity and uniformity of results. The Spark, the latest addition to Tecan's multimode reader portfolio, offers a number of features to address these demands and ensure optimal assay performance. The instrument's Fusion Optics combine the sensitivity of filters and the flexibility of monochromators in one instrument, allowing the operator to freely choose which optical system to use on the excitation and emission sides for the best possible results quality. HTRF measurements on the Spark can be performed with any combination of filter- and monochromator-based optics; filters are recommended for best possible HTRF measurement sensitivity and uniformity while monochromator-based measurements allow assay development and optimization using the instrument's tuneable bandwidth capabilities. Automated Z positioning and integrated dichroic mirrors complete the Spark's sophisticated optical system.

This application note assesses the performance of HTRF assays on the Spark, with a focus on the best possible combination of optics for sensitive, uniform and reproducible measurements. Recommended settings for selected HTRF assay kits – Reader Control, Human TNF- α and cAMP HiRange – are also included.

MATERIAL & METHODS

A Spark multimode reader was tested in combination with three HTRF assays: the HTRF Reader Control Kit (RCK), the Human TNF- α assay and the cAMP HiRange assay. While the RCK is used to confirm the general applicability of a reader for HTRF assays regarding several parameters, the TNF-alpha assays is suitable to assess the sensitivity of a reader to detect very low HTRF signals, and the cAMP assay is commonly used to determine the dynamic range of a test instrument. All kits were used according to the manufacturer's instructions.

Materials

- Spark multimode reader (Tecan, Austria)
- HTRF Reader Control Kit (Cisbio Bioassays, France)
- HTRF Human TNF- α Assay Kit (Cisbio Bioassays, France)
- HTRF cAMP HiRange Assay Kit (Cisbio Bioassays, France)
- 96-well half area plates (white) (Greiner Bio-One, Germany)
- 384-well small volume plates (white and black) (Greiner Bio-One, Germany)

HTRF RCK

The HTRF RCK is used to confirm the general suitability of a reader for HTRF assays, as well as set up and performance validation. It contains all the reagents and buffers necessary to assess the performance of an individual reader, and can also be used for the comparison of different instruments.

Human TNF- α Assay Kit

Tumor necrosis factor-alpha (TNF- α) is a cytokine secreted by activated macrophages and monocytes, and is involved in systemic inflammation as part of the acute phase immune response. The assay kit allows quantification of TNF- α in cells or supernatants by the antibody-mediated recognition of two distinct epitopes tagged with HTRF donor and acceptor fluorophores. The HTRF ratio increases linearly with the TNF- α concentration, making it ideally suited to assessing the sensitivity of a reader to very low intensity signals.

cAMP HiRange Assay Kit

Cyclic adenosine 3',5'-monophosphate (cAMP) is one of the most important intracellular messengers, and is involved in numerous biological processes, such as the activation of protein kinase or ion channels. This kit is designed for the direct quantification of cAMP in suspension or adherent cells using a competitive immunoassay, enabling the measurement of the agonist and antagonist effects on GPCRs (G-protein coupled receptors) in different cell lines. The HTRF signal is inversely proportional to the cAMP concentration in the sample, and this assay is commonly used to determine the dynamic range of a test instrument.

For most HTRF measurements, a combination of 100 μ s lag time and 400 μ s integration time offers the best sensitivity, dynamic range and lowest measurement



variation. However, it can be useful to modify the lag and integration time settings to further improve the assay window or the sensitivity. The flash number also directly influences the measurement CVs; lower flash numbers can be used to accelerate measurements, while higher flash numbers provide the best possible sensitivity and CVs. For the filter-based optics on both the excitation and emission sides (FF), the optimal flash number is 5-10 flashes per well, while 75-100 flashes is optimal for entirely monochromator-based measurements (MM). The validated measurement settings are summarized in Table 1 below.

Parameter	Setting
Label 1	Donor
Measurement mode	TR Fluorescence Intensity
EX wavelength	320 (25) nm (Filter) / 320 (20) nm (Mono)
EM wavelength	620 (10) nm (Filter) / 620 (10) nm (Mono)
Lag time	100 ms
Integration time	400 ms
Flashes	Recommended: 3-10 (Filter) / 50-100 (Mono)
Gain	Optimal
Mirror	Dichroic 510 or 'automatic'
Z position	Calculated from well
Label 2	Acceptor
Measurement mode	TR Fluorescence Intensity
EX wavelength	320 (25) nm / 320 (20) nm (Mono)
EM wavelength	665 (8) nm / 665 (10) nm (Mono)
Lag time	100 ms
Integration time	400 ms
Flashes	Recommended: 3-10 (Filter) / 50-100 (Mono)
Gain	Optimal
Mirror	Dichroic 510 or 'automatic'
Z position	As Label 1

Table 1: Recommended HTRF measurement settings for the Spark.

Generally, the mirror selection should be set to 'automatic', and the Z position should be calculated from any well containing the whole HTRF chemistry (ie. donor and acceptor). The gain should also be set to 'optimal' for both labels. To achieve fastest-possible measurement speed, mirror selection and Z positioning can be optimized in a pre-measurement, then set manually.

RESULTS

The results summarized here were obtained with the Spark's filter-based optics for optimal sensitivity. Other possible optics combinations were also tested (results not shown) with the available HTRF assays, and based on the results seen with the three assays the HTRF performance observed with the different optics combinations can be ranked as follows:

1. FF in white plates
2. MF in white plates
3. FF in black plates
4. FM in white plates
5. MM in white plates
6. MF in black plates
7. FM in black plates

The combination MM in black plates is not certified for HTRF applications

For convenience only FF data for the RCK, the TNF- α and the cAMP assays are shown in this application note.

HTRF Reader Control Kit

The Spark exhibited optimal performance for the HTRF RCK in white 96-well half area plates (supplied with kit). Using 10 flashes per well, only minimal variation in results was observed, as indicated by exceptionally low CVs in all standard and control replicates (Table 2).

	Criteria	Result
% CV Std 0	≤ 10	0.9
% CV Low Control	≤ 10	1.0
% CV High Control	≤ 10	1.8
ΔF Low (%)	≥ 15	28
ΔF High (%)	≥ 600	843
S/B	≥ 40	345

Table 2: Performance parameters of the RCK measured in white 96-well half area plates (10 flashes).

To further assess the instrument performance, the RCK was measured in white (Table 3) and black (Table 4) 384-well low-volume plates (filling volume: 20 μ l/well).



Flashes	1	10	25	50	75	100
S/B	676	623	627	629	600	562
% CV Std 0	7.7	1.8	1.4	1.1	0.9	0.7
ΔF Low (%)	37	41	41	43	41	44
ΔF High (%)	1,082	1,138	1,138	1,151	1,166	1,245
% CV Low	4.8	3.9	2.6	3.0	3.3	3.0
% CV High	3.1	2.8	2.6	2.7	2.7	2.4
Z' value	0.92	0.92	0.92	0.92	0.92	0.93

Table 3: Performance parameters of the RCK measured in white 384-well low-volume plates (10 flashes).

Flashes	1	10	25	50	75	100
S/B	201	209	199	203	207	207
% CV Std 0	13	4.6	3.9	2.7	3.4	3.1
ΔF Low (%)	28	32	36	33	30	32
ΔF High (%)	944	902	920	928	921	950
% CV Low	11.8	5.5	1.7	3.3	1.7	1.3
% CV High	7.9	5.2	4.4	3.8	3.4	2.4
Z' value	0.78	0.85	0.85	0.88	0.89	0.92

Table 4: Performance parameters of the RCK measured in black 384-well low-volume plates (10 flashes).

The results clearly show that increasing the flash number improves the measurement CVs and Z' values. A higher flash number is therefore recommended if best possible measurement sensitivity is required, while the flash number can be reduced to improve measurement speed.

Human TNF- α Kit

The Human TNF- α Kit requires exceptional reader sensitivity, with a detection limit of 20 pg/ml required to meet the manufacturer's validation criteria. The sensitivity of this assay is calculated using four calibrator solutions, and measurements performed with the Spark showed excellent linearity, as indicated by correlation coefficients (R^2 values) of >0.999 in both white and black plates (Figure 1). The MM optics also delivered a good linearity, with a R^2 value of 0.996 (data not shown).

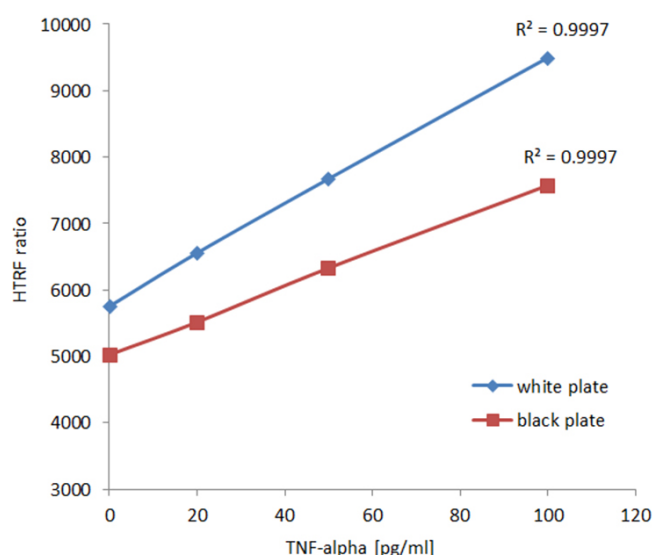


Figure 1: Linearity of the TNF- α dilution series using black and white plates.

As summarized in Table 5, even with just a single flash, the detection limit in white plates is exceptionally low, and easily meets the sensitivity criteria of the assay kit (20 pg/ml). When black plates are used, the flash number should be set to at least 25 to achieve the required sensitivity. With the MM optics, a minimum flash number of 50 is recommended to achieve reliable measurement sensitivity in white plates. Importantly, the MM optics are not certified for HTRF measurements in black plates, as the light levels are too low for acceptable assay performance.

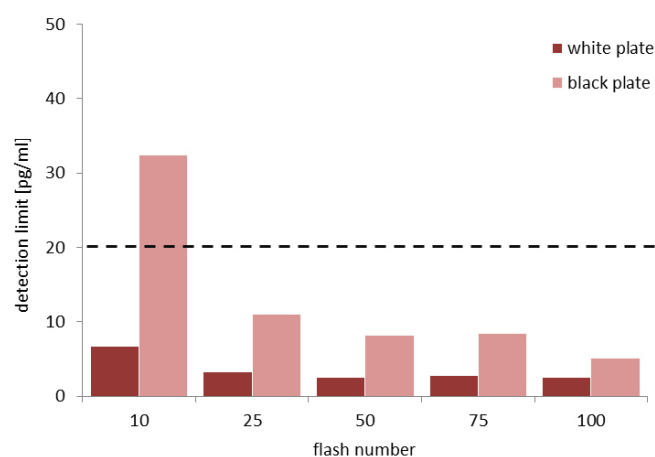


Figure 2: TNF- α detection limits with different flash numbers in black and white plates.

The detailed results obtained in white and black plates with varying flash numbers are summarized in Tables 5 and 6.



Flashes	1	10	25	50	75	100
Gain 620/665	127/185	127/184	127/184	127/185	127/184	127/184
deltaF Cal 1	16	13	14	14	15	14
deltaF Cal 2	33	32	34	33	37	35
deltaF Cal 3	64	64	68	65	72	68
CV Std 0	4.6	2.2	1.3	1.1	1.2	1.1
R ² value	0.9979	1.0000	0.9999	0.9997	0.9998	0.9997
DL [pg/ml]	12.2	6.7	3.3	2.6	2.8	2.6

Table 5: TNF- α assay performance with different flash numbers in white plates

Flashes	1	10	25	50	75	100
Gain 620/665	163/223	164/224	163/223	163/223	162/222	162/221
deltaF Cal 1	12	8	9	10	11	12
deltaF Cal 2	24	22	25	26	27	30
deltaF Cal 3	50	49	50	51	53	57
CV Std 0	16.1	7.4	2.7	2.1	2.3	1.7
R ² value	0.9969	0.9955	0.9997	0.9997	1.0000	0.9996
DL [pg/ml]	64.1	32.4	11	8.2	8.4	5.1

Table 6: TNF- α assay performance with different flash numbers in black plates.

cAMP HiRange Assay

As shown in Figure 3, the ΔF values obtained with the cAMP dilution series are inversely proportional to the cAMP concentration. This results in the sigmoidal curve that is typical of competitive assays. The detailed measurement results are summarized in Table 7. In each case, a sufficient dynamic range with a signal-to-blank ratio of ≥ 20 was achieved, with EC₅₀ values of 13.3 and 14.2 nM in white and black plates, respectively.

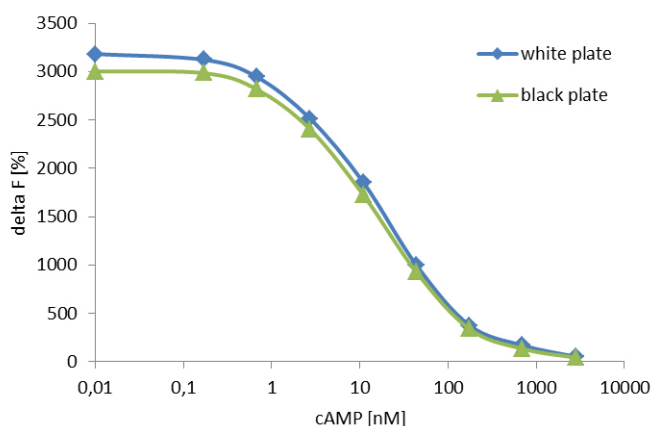


Figure 3: Competitive cAMP curves measured in both black and white plates (100 flashes).

	White	Black
S/N	34.7	27.7
EC ₅₀ [nM]	13.3	14.2
Gain 620/665	129/131	163/167

Table 7: Quality parameters of cAMP assay measured in white and black plates with the 50 flashes.

Lastly, the fastest possible measurement times (ie. with manual mirror selection and pre-optimized gain and Z position values) for a full 384-well plate were determined for the FF and MM optics combination, as shown in Table 8.

Flashes	1	10	25	50	75	100
FF 384-well	00:31	2:06	4:02	7:17	10:32	13:44
MM 384-well	00:37	2:11	4:08	7:23	10:37	13:50

Table 8: HTRF measurement times with different flash numbers in 384-well plates (all wells).

CONCLUSION

The Spark, with its unique Fusion Optics, provides a versatile, high performance detection system for HTRF assays. As shown in this application note, it meets the kit manufacturer's sensitivity, uniformity and dynamic range criteria for HTRF-compatible instruments, and is therefore certified for the HTRF assay technology. The system's filter-based optics offer the best possible sensitivity and uniformity, while the monochromator-based optics allow assay optimization by combining variable excitation and/or emission wavelength selection with acceptable sensitivity. When working with monochromators, we recommend that experiments are performed in white microplates, using at least 50 flashes per measurement, and only working within the linear detection range of the instrument, indicated by a gain value of 80-230. Although the kits tested here all use a europium donor, reliable and sensitive quantification can also be performed using HTRF assays employing a terbium donor, with both red and green acceptors (data not shown).

ACKNOWLEDGEMENTS

We would like to thank Sandrine Cabirol and François Degorce from Cisbio Bioassays for their support during the instrument validation.

ABBREVIATIONS

cAMP	cyclic AMP
DL	detection limit
EC	effective concentration
Em	emission
Ex	excitation
FF	filter/filter
MM	monochromator/monochromator
RCK	Reader Control Kit
S/N	signal-to-noise
TNF	tumor necrosis factor

About the author

Dr. Katrin Flatscher is an application scientist at Tecan Austria. She studied molecular biology at the University of Salzburg and focused on cell biology and immunology research during her PhD. She joined Tecan in 2007 and has been involved in the development of the Infinite as well as the Spark multimode reader series.

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