Application Note

MEASURING HTRF® ASSAYS WITH THE TRISTAR 5 MULTIMODE READER

Abstract

HTRF® assays are homogeneous assays which provide high sensitivity and reliability in many different immunoassays and methods measuring molecular interactions. However, they require a high-performance microplate reader that delivers high sensitivity. In this application note, the performance of the Tristar 5 multimode microplate reader configured in filter mode with red photomultiplier (Id.Nr: 69185-25, 69185-45 and 69185-50), is tested in 4 different HTRF® assays: TNFα and cAMP Gs HiRange (europium donor/red acceptor), IP-One Gq (Terbium donor/red acceptor) and cAMP HTPlex (Terbium donor/green acceptor). We conclude that the Tristar 5 performed well in all of them, as all required performance indicators are met.

Introduction

Methods able to measure molecular interactions are of high importance in many areas of research. However, many methods, such as ELISA, which measures the interaction between an antigen and an antibody, require several binding and washing steps, which make them lengthy and laborious. HTRF® technology (Homogeneous Time-Resolved Fluorescence) allows the measurement of molecular interactions without any washing steps, and this has been used to develop many assays which are fast and easy to perform, delivering high throughput and reliability.

HTRF® is based on Time-Resolved Fluorescence (TRF) and Fluorescence Resonance Energy Transfer (FRET). Two of the molecules of interest are labelled, one with the donor, one with the acceptor. When the donor and the acceptor are in close vicinity, FRET happens, and the signal of the acceptor can be detected. In HTRF® a lanthanide cryptate is used as donor and fluorescence is measured using time-resolved techniques, which greatly reduces background and improves sensitivity compared to non-time-resolved fluorescent methods. In addition, HTRF® implements a ratiometric measurement to correct for well-to-well variability and signal quenching from assay components and media, which improves the reliability of the method. HTRF® technology offers a high performance for immunoassays, protein-protein interaction methods, receptor binding assays, biomarkers detection and more.

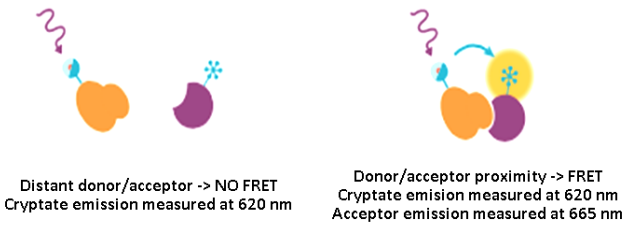

Figure 1: schematic representation of the principle of HTRF® assays.

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HTRF® assays come with many advantages but measuring them requires a high-performance microplate reader which can meet the demanding requirements of this technology. The Tristar 5 ONE-4-ALL Optics have been optimised to combine the stability and user-friendliness of a multi-modal optical system with the sensitivity and versatility of dedicated optical devices. In this application note, the performance of the Tristar 5 multimode microplate reader to perform HTRF® assays is assessed, and measurement settings are optimized for each donor/acceptor pair.

Tristar 5 Multimode Microplate Reader

Flexibility and sensitivity whenever you need it

The Tristar 5 is a modular high-performance microplate reader equipped with FlexTec Optics, offering you the best of two worlds: independent, user-selectable filters and monochromators on both, the excitation and emission side, for any measurement. This guarantees both, flexibility, and sensitivity whenever you need it.

The Tristar 5 Multimode Microplate Reader provides you with application flexibility for today, tomorrow, and beyond to master your changing plate reading applications:

- High sensitivity luminescence
- BRET
- Absorbance (UV/VIS)
- Fluorescence
- FRET
- TRF, TR-FRET and HTRF®
- AlphaScreen®
- FP

To meet your compliance requirements, a set of validation tools and optional software providing 21 CFR part 11 compliance are available.

Materials

- Tristar 5 Multimode Microplate Reader from Berthold Technologies (Id. Nr. 69185-25).
- HTRF® detection technology for the Tristar series (Id. Nr. 62772).
- Certus dispenser from Gyger.
- 384-well, microplates, small volume from Greiner (White #784075, Black #784076).
- Eppendorf tubes of various volumes.
- Pipettes and pipette tips (various volumes).

The following kits were tested:

- Reader control kit from PerkinElmer (Ref # 62RCLPEA)
- Human TNFα kit from PerkinElmer (Ref # 62HTNFAPEG)
- cAMP Gs HiRange kit from PerkinElmer (Ref # 62AM6PEB)
- IP-One Gq kit from PerkinElmer (Ref # 62IPAPEB).
- cAMP HTPlex-assay from PerkinElmer (not commercially available).

Reader Control Kit (RCK)

The HTRF® Reader Control Kit provides an easy and convenient way to assess the overall performance of a microplate reader. Thanks to the stability of HTRF® signal, the same assay plate can be used to check the performance of an individual reader with different setup configurations and allows as well to compare different instruments.

Human TNF alpha kit

TNF alpha is a cytokine produced by macrophages, T cells, and neutrophils. TNF alpha acts as a chemoattractant for neutrophils and promotes infiltration and activation at inflammation sites. TNF alpha is involved in several cancer types for its ability to promote angiogenesis and cell proliferation, as well as to inhibit apoptosis. TNF alpha is detected in sandwich assay format. The HTRF® signal is proportional to the concentration of the analyte in the sample.

cAMP Gs HiRange kit

This kit detects changes of cAMP accumulation in response to Gs coupled GPCR activation or inhibition and can be used also to monitor the phosphodiesterase enzyme activity. The principle of this assay is based on a competitive format where the cAMP from sample competes with cAMP labelled with acceptor dye. The HTRF® signal is inversely proportional to the cAMP concentration.

IP-One Gq kit

This kit detects the accumulation of inositol monophosphate, a stable downstream metabolite of IP3 induced by activation of a phospholipase C (PLC) cascade. This kit allows direct characterization of all types of compounds acting on Gq-coupled receptors in both adherent and suspension cells. It is a competitive assay where native IP-One competes with IP-One with labelled acceptor dye. The HTRF® signal is inversely proportional to IP-one concentration in the sample.

Instrument settings

Different filter combinations were used for each donor/acceptor combination:

Europium donor, red acceptor (TNFα, cAMP HiRange):

- Excitation: 320/40 (HTRF Eu cryptate)
- Emission (donor): 620/10uv (HTRF Eu cryptate)
- Emission (acceptor): 665/7uv (HTRF XL665/APC)

Terbium donor, red acceptor (IP-One):

- Excitation: 340/26 (HTRF Tb cryptate)
- Emission (donor): 620/10uv (HTRF Eu cryptate)
- Emission (acceptor): 665/7uv (HTRF XL665/APC)

Terbium donor, green acceptor (cAMP HTPlex)

- Excitation: 340/26 (HTRF Tb cryptate)
- Emission (Acceptor): 520/10uv (Green dye)
- Emission (Donor): 620/10uv (HTRF Eu cryptate)

Different settings were tested (see below). Optimal settings are summarized in Table 1, 2 and 3.

Methods

Reagents of all kits were prepared according to the manufacturer's instructions. Microplates were dispensed using the Certus dispenser. The Tristar 5 was used in filter mode in all tests.

The Reader Control Kit was used to confirm that the standard settings for the TriStar^2 S were suitable also for the Tristar 5. Tested settings were:

Measurement 1 (donor)

- Measurement mode: TRF Endpoint
- Delay before measurement: 0 s
- Counting time: 1.0 s
- Use filters
- Aperture: 2 – Rd 11
- Excitation filter: 320/40 (HTRF Eu cryptate)
- Excitation optic: 2 – Small filter 0.25 mm
- Emission filter: 620/10uv (HTRF Eu cryptate)
- Cycle time: 5000 µs
- Delay time: 100 µs
- Reading time: 300 µs
- Operation mode: by plate

Measurement 2 (acceptor)

- Measurement mode: TRF Endpoint
- Delay before measurement: 0 s
- Counting time: 1.0 s
- Use filters
- Aperture: 2 – Rd 11
- Excitation filter: 320/40 (HTRF Eu cryptate)
- Excitation optic: 2 – Small filter 0.25 mm
- Emission filter: 665/7uv (HTRF XL665/APC)
- Cycle time: 5000 µs
- Delay time: 100 µs
- Reading time: 300 µs
- Operation mode: by plate

Settings were further optimized for specific assays in subsequent tests.

For the optimization of the TNFα kit (Europium donor, red acceptor), 24 replicates of 4 calibrators were measured. The concentrations of the calibrators were 0, 20, 50 and 100 pg/mL TNFα. Following the manufacturer's instructions, 16 µL of each calibrator were pipetted per well, and 4 µL of pre-mixed TNFα antibody working solution were added. The microplate was sealed and incubated for 2 h at room temperature before measurement.

For the optimization of the cAMP Gs High Range kit (Europium donor, red acceptor), a standard curve with the following concentrations was prepared in triplicate: 0, 1, 3, 11, 44, 175, 700, 2800 nM cAMP. Following the manufacturer's instructions, 5 µL of each standard were dispensed, and the following reagents were dispensed to each well: 5 µL Stimulation Buffer 1, 5 µL cAMP d2 reagent working solution and 5 µL of cAMP Eu Cryptate antibody working solution. The plate was sealed and incubated for 1 h at room temperature before measurement.

For the optimization of the IP-One kit (Terbium donor, red acceptor), a standard curve with the following concentrations was prepared in triplicate: 0, 2, 8, 30, 120, 481, 1925, 7700 nM IP1. Following the manufacturer's instructions, 14 µL of each standard were dispensed, and the following reagents were dispensed to each well: 3 µL IP1 d2 Reagent working solution and 3 µL IP1 Tb Cryptate

Antibody working solution. The plate was sealed and incubated for 1 h at room temperature before measurement.

For the optimization of the cAMP HTPlex assay (Terbium donor, green acceptor), a standard curve with the following concentrations was prepared in triplicate: 0, 1, 3, 11, 44, 175, 700, 2800 nM cAMP. Reagents were dispensed and the plate was incubated following the manufacturer's instructions.

The following settings were tested (not all combinations tested for all kits):

- Delay before measurement: 0, 0.1, 0.2 and 0.3 s
- Counting time: 1.0 and 1.2 s
- Aperture: 2 – Rd 11 and 3 – Rd 2
- Cycle time: 2000 and 5000 µs
- Delay time: 50, 75 and 100 µs
- Reading time: 300, 350 and 400 µs
- Excitation optic: 2 – Small filter 0.25 mm and 3 – Wide 0.45 mm

All other settings as indicated above for the Reader Control Kit.

For all kits, the ratio of the acceptor and donor emission was calculated as (Signal 665 nm/Signal 620 nm) x 10000.

Results

For the TNFα kit, the settings of the TriStar^2 S gave acceptable results, but using a delay of 0.2 s before measuring improved the detection limit by a 41%. This modification, combined with the use of 400 µs reading time instead of 300 µs, further improved the detection limit, reaching 4.9 pg/mL TNFα. The result is notably better than the <12.5 pg/mL required by the norm of the kit. The calibrator curve obtained with those settings is represented in Figure 2. Other settings tested did not improve the detection limit.

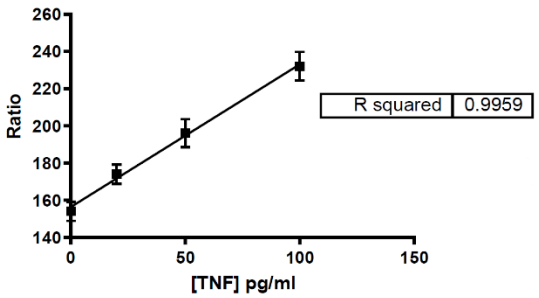


Figure 2. Calibrator curve of TNFα using the optimal settings for the Tristar 5.

For the cAMP Gs HiRange kit, as donor and acceptor are of the same type as in the TNFα kit, the optimal settings for TNFα were used, which delivered good results: S/N 31.1 (the norm requires S/N > 20) and EC50 9.3 nMf (nM final concentration; the norm requires EC50 < 25 nMf). Other settings tested, such as shorter Delay Times, did not improve the performance of the assay (Figure 3).

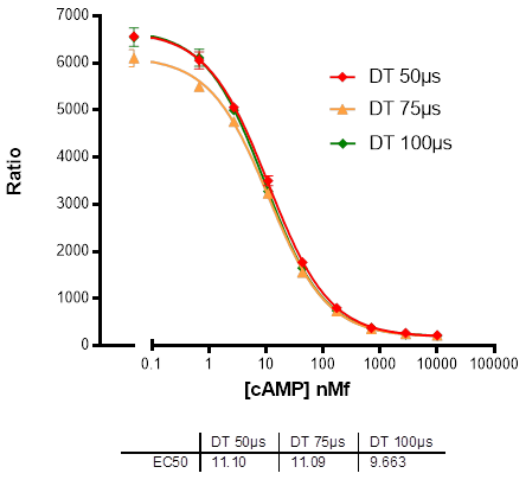
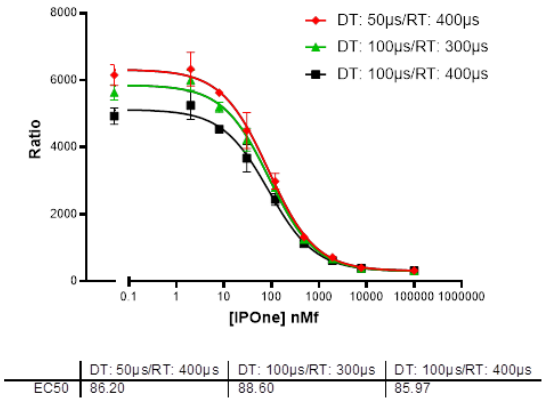


Figure 3. Standard curve of cAMP comparing the effect of different Delay Times.

In the case of the IP-One kit, a different dye combination is used (Terbium donor, red acceptor), and excitation uses a 340/26 filter instead of the

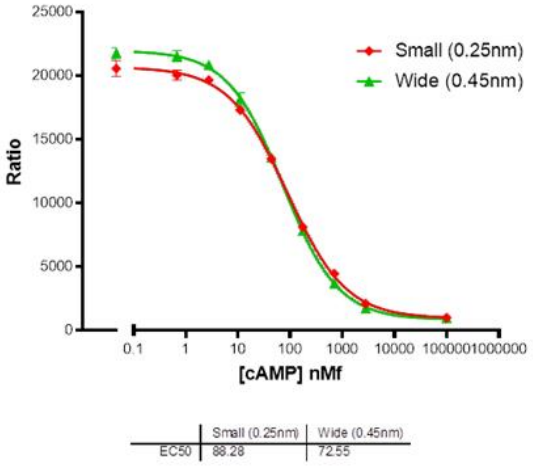
320/40 one. Optimal settings (see Table 2) were very similar to the ones used for TNFα and cAMP, except for Cycle Time (2000 µs) and Delay Time (50 µs). With those settings, an EC50 of 85.97 nMf was obtained (the norm requires EC50 < 150 nMf). A representative standard curve is shown in Figure 4.

For the cAMP HTPlex assay, the wide excitation optic provided the best performance (S/N 22.6, EC50 72.55 nMf; see Figure 5), which met the requirements of the norm (S/N > 15, EC50 < 75 nMf). Other settings tested, such as shorter reading or delay times, did not improve the performance of the assay. Optimal settings for this dye combination (Terbium donor, green acceptor) are summarized in Table 3.



	DT: 50µs/RT: 400µs	DT: 100µs/RT: 300µs	DT: 100µs/RT: 400µs
EC50	86.20	88.60	85.97

Figure 4. Standard curve of IP1 comparing the effect of different Delay Times (DT) and Reading Times (RT)



	Small (0.25nm)	Wide (0.45nm)
EC50	88.28	72.55

Figure 5. Standard curve of cAMP comparing the effect of different excitation optics.

With all kits the performance in black microplates was generally worse than in white ones and, in some cases, it didn't reach the norms (data not shown). Hence, HTRF® assays with the Tristar 5 must always be performed in white microplates.

Discussion and conclusions

The optimal settings for the predecessor model, the TriStar2 S, were used as a starting point. These settings provided valid results thanks to the high compatibility of the entire Tristar family, although HTRF® assays are very demanding. However, small modifications, such as using a short delay before measurement and slightly increasing the reading time (for the Europium donor/red acceptor combination) even improved the performance.

Performance of the Tristar 5 using white microplates was excellent, meeting all criteria of the kits tested.

In conclusion, the Tristar 5 Multimode Microplate Reader with its unique ONE-4-ALL Optics is well suited to measure HTRF® assays in white microplates using filters on both, the excitation, and the emission side.

Summary of optimal settings for each kit

Among 8 pre-configured models of Tristar 5 reader, 3 models are compatible and HTRF® certified, when used in filter mode:

- Research FL (ID: 69185-45)
- Research Plus FL (ID: 69185-50)
- Research Performance FL (ID: 69185-25)

ID is indicated on the label on the back of the reader.

The Tristar 5 operating software comes with pre-set ready-to-use parameter files for HTRF® measurements including the ratio calculation. The recommended settings are defined under the TR-Fluorescence protocol as described below:

Table 1: optimal settings for HTRF® kits using Europium donor and red acceptor

Setting	**Measurement 1 (donor)**	**Measurement 2 (acceptor)**
Measurement mode	TRF Endpoint	TRF Endpoint
Delay before measurement	0.2 s	0.2 s
Counting time	1.0 s	1.0 s
Use filters	Yes	Yes
Aperture	2 – Rd 11	2 – Rd 11
Excitation filter	320/40 (HTRF Eu cryptate)	320/40 (HTRF Eu cryptate)
Excitation optic	3 – Wide filter 0.45 mm	3 – Wide filter 0.45 mm
Emission filter	620/10uv (HTRF Eu cryptate)	665/7uv (HTRF XL665/APC)
Cycle time	5000 µs	5000 µs
Delay time	100 µs	100 µs
Reading time	400 µs	400 µs
Operation mode	by plate	by plate
Plate color	White plate only	

Table 2: optimal settings for HTRF® kits using Terbium donor and red acceptor

Setting	**Measurement 1 (donor)**	**Measurement 2 (acceptor)**
Measurement mode	TRF Endpoint	TRF Endpoint
Delay before measurement	0.2 s	0.2 s
Counting time	1.0 s	1.0 s
Use filters	Yes	Yes
Aperture	2 – Rd 11	2 – Rd 11
Excitation filter	340/26 (HTRF Tb cryptate)	340/26 (HTRF Tb cryptate)
Excitation optic	3 – Wide filter 0.45 mm	3 – Wide filter 0.45 mm
Emission filter	620/10uv (HTRF Eu cryptate)	665/7uv (HTRF XL665/APC)
Cycle time	2000 µs	2000 µs
Delay time	50 µs	50 µs
Reading time	400 µs	400 µs
Operation mode	by plate	by plate
Plate color	White plate	

Table 3: optimal settings for HTRF® kits using Terbium donor and green acceptor

Setting	**Measurement 1 (donor)**	**Measurement 2 (acceptor)**
Measurement mode	TRF Endpoint	TRF Endpoint
Delay before measurement	0.2 s	0.2 s
Counting time	1.0 s	1.0 s
Use filters	Yes	Yes
Aperture	2 – Rd 11	2 – Rd 11
Excitation filter	340/26 (HTRF Tb cryptate)	340/26 (HTRF Tb cryptate)
Excitation optic	3 – Wide filter 0.45 mm	3 – Wide filter 0.45 mm
Emission filter	520/10uv	620/10uv (HTRF Eu cryptate)
Cycle time	2000 µs	2000 µs
Delay time	50 µs	50 µs
Reading time	400 µs	400 µs
Operation mode	by plate	by plate
Plate color	White plate	

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