Molecular Devices

HTRF Human TNFα Assay on SpectraMax Paradigm Multi-Mode Microplate Reader

In this application note we show how the SpectraMax® Paradigm® Multi-Mode Microplate Reader is used to perform robust, no-wash cytokine assays with excellent Z' factors.

HTRF® is a versatile technology developed by Cisbio Bioassays for detecting biomolecular interactions[1]. It combines fluorescence resonance energy transfer (FRET) technology with time-resolved (TR) measurement of fluorescence, allowing elimination of short-lived background fluorescence. The assay uses donor and acceptor fluorophores. When donor and acceptor are close enough to each other, excitation of the donor by an energy source (e.g., a flash lamp) triggers an energy transfer to the acceptor, which in turn emits specific fluorescence at a given wavelength.

HTRF uses four specific fluorophores that can be combined to form compatible donor-acceptor TR-FRET pairs. The donors are europium cryptate (Eu3+) and Terbium (Lumi4™-Tb) cryptate, whose long-lived fluorescence enables their use in time-resolved fluorescence assays[1]. Two acceptors have been developed for use in HTRF assays, XL665 and d2. Both have excitation spectra that overlap the emission spectrum of the HTRF donors. Each has an emission peak at 665 nm that falls within a region where the donor does not emit, or emits very weakly. The original HTRF acceptor, XL665, is a phycobiliprotein pigment purified from red algae. A second generation acceptor, d2, is a modified allophycocyanin that is 100 times smaller than XL665 and was developed to alleviate steric hindrance problems that may occur with XL665-based assays.

Cytokines like tumor necrosis factor alpha (TNFα) are of particular interest in the fields of oncology, infectious disease, allergies, and autoimmune diseases. Disturbances in cytokine regulation are known to cause inappropriate or ineffective immune responses.

The Human TNFα assay kit enables direct quantitative determination of TNFα. It uses a sandwich immunoassay involving two specific antibodies, one anti-TNFα labeled with Eu-Cryptate and the second anti-TNFα labeled with XL665 or d2. In the presence of TNFα, both antibodies bind TNFα, bringing both labels into close proximity so that TR-FRET occurs (Figure 1). Signal is proportional to the concentration of antigen in the sample.

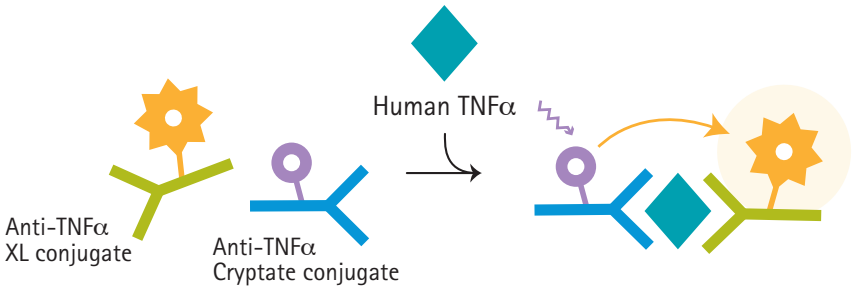
Benefits

- Highly robust homogeneous assay
- Z' factor ≥ 0.9
- Streamlined and stable for HTS
- Faster time to results with SoftMax Pro Software protocols

Materials

- Human TNFα kit, 1000 tests (Cisbio P/N 62TNFPEB)
- Black and white low-volume 384-well microplates (Greiner P/N 784076 and 784075)
- SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices)
- HTRF Detection Cartridge (Molecular Devices P/N 0200-7011)

Figure 1. Human TNFα assay principle



Antibodies labeled with donor and acceptor fluorophores bind to TNFα, bringing the labels into close proximity and enabling TR-FRET.

Methods

Human TNFα standards ranging from 20 to 2000 pg/mL were prepared as indicated in the kit product insert. A positive assay control consisting of free human TNFα, and a negative control without human TNFα (no TR-FRET), were included to confirm assay activity and aid in accurate calculation of results.

Reagents were dispensed in a final volume of 20 µL per well as indicated in Table 1.

Data Analysis

Analysis of HTRF assays uses Cisbio's patented ratiometric reduction method based on the two emission wavelengths detected. Donor emission at 616 nm is used as an internal reference, while acceptor emission at 665 nm is used as an indicator of the biological reaction being assayed. This ratiometric measurement reduces well-to-well variation and eliminates compound interference. Delta F, calculated in step 4 below, reflects signal to background of the assay and is useful for inter-assay comparisons.

Results are calculated from the 665 nm/616 nm ratio and expressed in Delta F as follows:

1. $\text{Ratio} = \frac{\text{Emission}_{665nm}}{\text{Emission}_{616nm}} \times 10^4$

2. $\text{Mean Ratio} = \frac{\Sigma\text{ratios}}{2}$

3. $\text{CV} = \frac{\text{Std deviation}}{\text{Mean ratio}} \times 100$

4. $\text{Delta F} = \frac{\text{Standard or sample Ratio} - \text{Ratio}_{neg}}{\text{Ratio}_{neg}} \times 100$

(Ratio_{neg} = Ratio of negative control)

Z' factor values were calculated from the negative control and 2000 pg/mL standard[2].

Data were generated and analyzed using SoftMax® Pro Software, which contains several preconfigured HTRF protocols to simplify detection and analysis.

Table 1. Assay setup for a 384-well low-volume plate

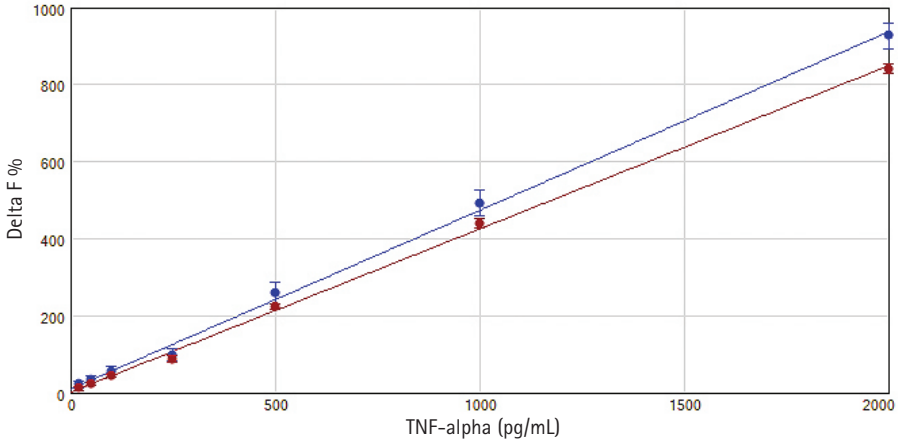
Standard curve	Negative control	Assay control
10 µL standard	10 µL diluent	10 µL TNFα control
5 µL XL665 conjugate		
5 µL Cryptate conjugate		

The plate was covered, incubated for three hours at room temperature, then time resolved fluorescence was measured on the SpectraMax Paradigm reader with HTRF Detection Cartridge (see Table 2 for instrument settings).

Table 2. Optimized instrument settings for SpectraMax Paradigm reader with HTRF Detection Cartridge

Optical Configuration	HTRF Detection Cartridge
Read Mode	TR-FRET
Read Type	Endpoint
Wavelengths	Ex 340 nm Em 616 nm Em 665 nm
PMT and Optics	Number of Pulses: 30 Excitation Time: 0.05 ms Measurement Delay: 0.03 ms Integration Time: 0.2 ms

Figure 2. HTRF Human TNFα calibration curve



HTRF Human TNFα calibration curve measured on the SpectraMax Paradigm reader. Blue circles: black plate; red circles: white plate. The r^2 value for each plot is 1.00.

Results

The SpectraMax Paradigm reader has read height and microplate optimization features, which allow users to easily determine the optimal read height and microplate dimensions, increasing the assay dynamic range and sensitivity. Both optimizations were performed for each Greiner plate. Data were expressed as Delta F % (DF %) and plotted against human TNFα concentrations (Figure 2). Best results were obtained with 30 pulses, delay of 0.03 ms and integration time of 0.2 ms (Table 2).

Similar results were obtained using the low-volume white and black 384-well microplates. DF% values ranged from 21 to 927 with the black plate and from 7 to 810 with the white plate. Although the assay window was larger with the black plate, the Z' factor for the white plate was 0.95, compared to 0.86 for the black plate, indicating slightly better overall assay performance[2].

Conclusion

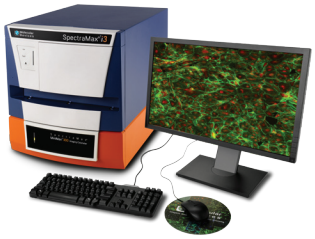
The SpectraMax Paradigm reader with HTRF Detection Cartridge offers high-throughput screening capability with excellent read times and Z' factors. Simultaneous dual emission detection enables read times of only 2:17 for an entire 384-well plate. Sensitivity and dynamic range are demonstrated by the high Z' factors obtained on the SpectraMax Paradigm reader. This assay may also be detected on the SpectraMax® i3 and SpectraMax® M5e Multi-Mode Microplate Readers. Data acquisition and analysis are simplified using SoftMax Pro Software with preconfigured HTRF protocols.

References

1. http://www.htrf.com/htrf-technology
2. Zhang, J. H., Chung, T. D. Y., and Oldenburg, K. R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomolecular Screening* 4(2): 67-73.

Compatible with these Molecular Devices systems

SpectraMax Paradigm
Multi-Mode Microplate Reader



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Microplate Reader

SpectraMax M5e Multi-Mode
Microplate Reader

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