APPLICATION NOTE

HTRF cAMP HiRange Assay on SpectraMax Multi-Mode Microplate Readers

Introduction

In this application note we show how the SpectraMax® i3, SpectraMax® Paradigm®, and SpectraMax® M5e Multi-Mode Microplate Readers are used to perform robust, high-throughput HTRF® assays with excellent Z' factors and highly reproducible EC_{50} values.

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.

The HTRF cAMP HiRange kit enables quantitation of cyclic AMP (cAMP, cyclic adenosine 3', 5'–monophosphate) in cellular samples. cAMP is a key second messenger in G protein-coupled receptor (GPCR) signaling. Upon ligand binding to a GPCR, a conformational change occurs, activating the receptor and in turn activating a G protein. Further signal transduction depends on the type of G protein activated. Activation of G_s leads to upregulation of cAMP by adenylate cyclase. Free cAMP produced by cells competes with d2-labeled cAMP for binding to the anti-cAMP cryptate, so an increase in cellular cAMP leads to a decrease in FRET, which is detectable as a decrease in the fluorescence emitted at 665 nm (Figure 1).

Benefits

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

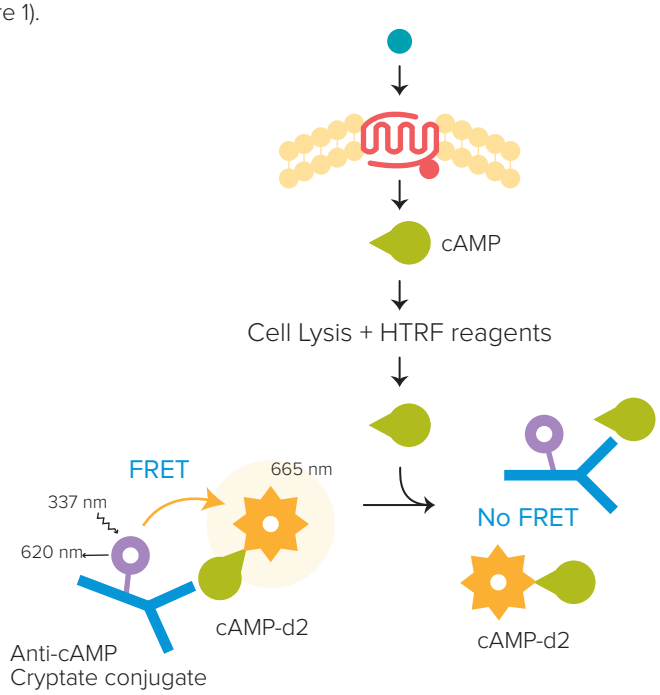

Figure 1. cAMP assay principle. Unlabeled cAMP produced by cells competes with d2-labeled cAMP for binding to anti-cAMP cryptate conjugate. Thus an increase in cellular cAMP leads to a decrease in FRET.

Materials

- cAMP HiRange 1000 tests (Cisbio P/N 62AM6PEB)
- Black and white low volume 384-well microplates (Greiner P/N 784076 and 784075)
- SpectraMax i3 Multi-Mode Microplate Reader (Molecular Devices)
- SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices)
- SpectraMax M5e Multi-Mode Microplate Reader (Molecular Devices)
- HTRF Detection Cartridge (Molecular Devices P/N 0200-7011)

Negative control	Positive control	Standard curve	Assay control
5 µL diluent	5 µL diluent	5 µL cAMP standard	5 µL cAMP control
5 µL diluent			
5 µL conjugate & lysis buffer	5 µL cAMP-d2		
5 µL anti-cAMP-Cryptate			

Table 1. Assay setup for a 384-well low-volume plate. The plate was covered and incubated for one hour at room temperature. Time-resolved fluorescence was measured on the SpectraMax Multi-Mode Microplate Readers (see Table 2 for instrument settings). Both Microplate Optimization and Read Height Adjustment were performed on the SpectraMax i3 and SpectraMax Paradigm readers to ensure optimal assay sensitivity and dynamic range.

Methods

The HTRF cAMP HiRange kit was provided by Cisbio Bioassays. cAMP standards with final concentrations ranging from 0.17 nM to 2800 nM were prepared as indicated in the cAMP HiRange HTRF package insert. A positive control without cAMP (maximum FRET) and a negative control without cAMP or cAMP-d2 were included. Reagents were dispensed in a final volume of 20 µL per well as indicated in Table 1.

SpectraMax i3 and SpectraMax Paradigm	
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: 0.02 ms Integration Time: 0.2 ms Read Height: 7.5-7.7 mm*

SpectraMax M5e	
Read Mode	TRF
Read Type	Endpoint
Wavelengths	Ex 314 nm Em 620 nm Em cutoff 570 nm Em 665 nm Em cutoff 630 nm
TRF Settings	Integration Delay: 50 µs Integration Time: 400 µs
PMT and Optics	Flashes per read: 100

*Optimal read height depends upon the microplate, assay volume, and reader optics.

Table 2. Optimized instrument settings for SpectraMax i3, SpectraMax Paradigm, and SpectraMax M5e readers.

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

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)

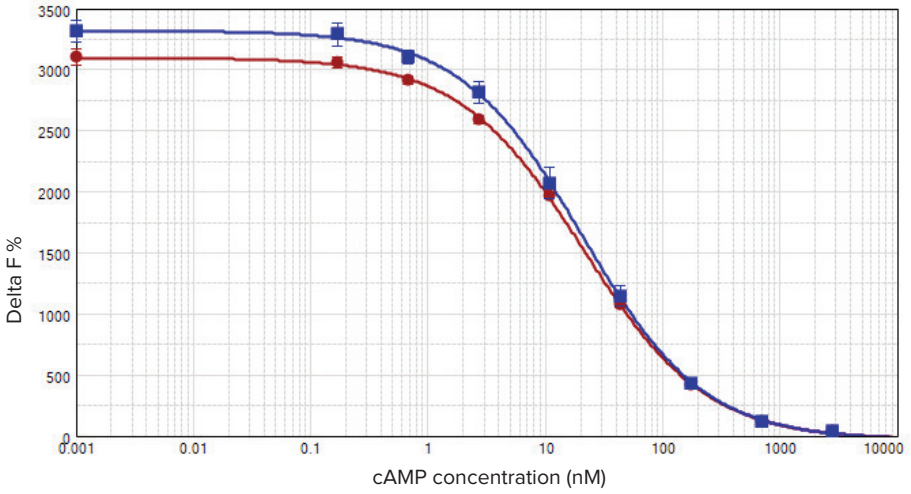

Figure 2. Comparison of white and black microplates. HTRF cAMP calibration curves measured on the SpectraMax Paradigm reader. Red circles: Greiner white plate; blue squares: Greiner black plate. In this example, using a black plate increased the assay window from 3020 to 3253 (units of Delta F%).

Z' factor values were calculated using the negative (no cAMP, no cAMP-d2) and positive (no cAMP) controls[2].

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

Measurement	SpectraMax i3 reader	SpectraMax Paradigm reader	SpectraMax M5e reader
cAMP EC_{50} (nM)	19.1	19.0	17.5
Z' factor	0.92	0.90	0.90
Read time (384 wells)	5:03	2:17	14:00

Table 3. Results summary for cAMP HiRange standard curve

Results

Data were analyzed as described above and graphed with SoftMax Pro Software using a 4-parameter curve fit. Best results were obtained with the reader settings indicated in Table 2. When delay time, integration time, and number of pulses were increased, a noticeable decrease in DF % was observed. An increase in assay window from 3020 to 3253 was obtained using a black low-volume 384-well microplate, compared to a white microplate of the same design (Figure 2). However, Z' factors, which take into account both assay window and standard deviation of positive and negative controls, and EC_{50} values were very similar for both plate types (Table 3). (Note: when using a SpectraMax M5e Multi-Mode Microplate Reader, white plates are required.)

SpectraMax i3 and SpectraMax Paradigm readers produced nearly identical results when compared side by side with the same assay plate (Figure 3). For this test, a white microplate was used. Assay windows were 3004 and 3051, and Z' factors were 0.92 and 0.90, respectively. EC_{50} values were 19.1 nM and 20.0 nM, comparable to published values (Table 3.) Similar Z' factors and EC_{50} values are obtained with the SpectraMax M5e Multi-Mode Microplate Reader.

Read time on the SpectraMax Paradigm reader is faster than on the other readers due to its dual PMT configuration that enables both HTRF emission wavelengths to be read simultaneously (Table 3).

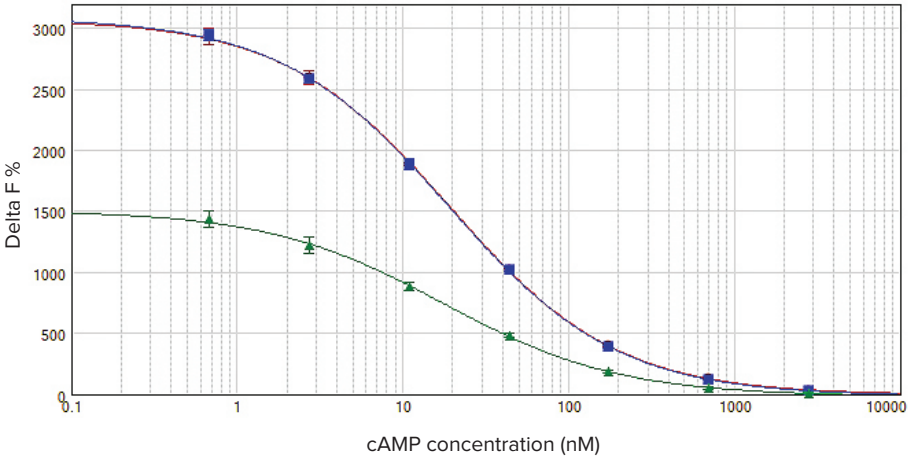

Figure 3. HTRF cAMP calibration curve. HTRF cAMP calibration curve measured on the SpectraMax i3 (red circles), SpectraMax Paradigm (blue squares), and SpectraMax M5e (green triangles) readers. Results on SpectraMax i3 and SpectraMax Paradigm readers were virtually identical. The assay window for the SpectraMax M5e reader was smaller but the assay quality was excellent.

Conclusion

The SpectraMax i3 and SpectraMax Paradigm readers can be equipped with an HTRF-certified detection cartridge with high-throughput screening capabilities. When used on the SpectraMax Paradigm reader, this cartridge offers simultaneous dual emission detection that enables faster read times. On all three readers, excellent Z' factor values for the cAMP HiRange assay demonstrate the robust assay performance of these multi-mode systems. 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 i3x Multi-Mode Microplate Reader

SpectraMax Paradigm Multi-Mode Microplate Reader

SpectraMax M5e Multi-Mode Microplate Reader

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