APPLICATION NOTE

Detect GPCR activity with the cAMP-G_s HiRange HTRF assay

Caroline Cardonnel | Sr. Applications Scientist | Molecular Devices

Benefits

- **Assured instrument performance with certified HTRF compatibility**
- **Highly robust, homogeneous assay for HTS**
- **Faster time to results with preconfigured SoftMax Pro Software protocols**

Introduction

HTRF® is a versatile technology developed by Cisbio for detecting biomolecular interactions. 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, which are used to label proteins or other biomolecules whose binding is to be studied. When the two biomolecules are bound to each other, the donor and acceptor are brought close together. 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 (Eu^{3+}) and terbium cryptate (Lumi4™-Tb), whose long-lived fluorescence enables their use in time-resolved fluorescence assays. 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 with an emission peak at 665 nm that falls within a region where the donor does not emit significantly. The original HTRF acceptor, XL665, is a phycobiliprotein pigment purified from red algae. The 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 protein 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).

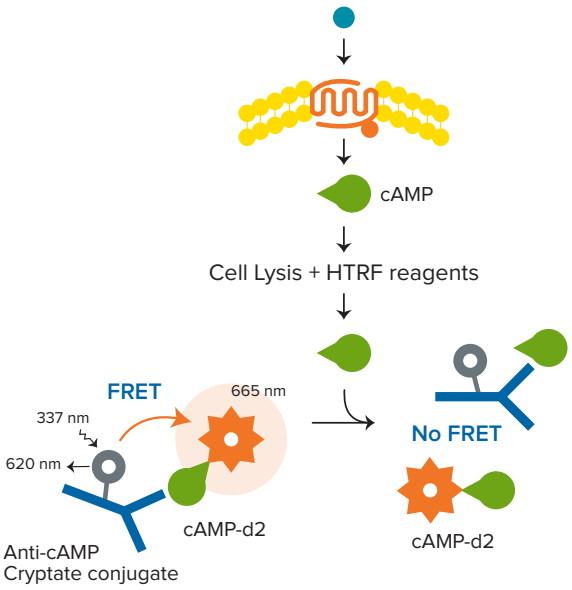

Figure 1. cAMP assay principle. Unlabeled cAMP produced by cells competes with d2-labeled cAMP for binding to anti-cAMP cryptate conjugate. Thus an increase in cellular cAMP leads to a decrease in FRET.

Here, we show how the SpectraMax® i3x and the SpectraMax® iD5 Multi-Mode Microplate Readers, both certified HTRF-compatible by Cisbio, are used to perform robust, no-wash assays for GPCR activity.

Materials

- cAMP G_s HiRange kit (1000 tests, Cisbio cat. #62AM6PEB)
- White low-volume 384-well microplates (Greiner cat. #784075)
- SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices cat. #i3x)
- SpectraMax iD5 Multi-Mode Microplate Reader (Molecular Devices cat. #iD5)
- HTRF Detection Cartridge (Molecular Devices cat. #0200-7011) for the SpectraMax i3x reader
- HTRF Detection System (Molecular Devices cat. #6590-0144, includes Enhanced TRF module and HTRF filters) for the SpectraMax iD5 reader

Methods

The cAMP G_s HiRange kit was provided by Cisbio. cAMP standards with final concentrations ranging from 0.17 nM to 2800 nM were prepared as indicated in the kit package insert. A positive control without cAMP (maximum FRET) and a negative control without cAMP or cAMP-d2 were included. Reagents were dispensed to a final volume of 20 µL per well in a low-volume 384-well microplate as indicated in Table 1.

The plate was covered and incubated for one hour at room temperature. Time-resolved fluorescence was measured on the SpectraMax i3x and SpectraMax iD5 readers using preconfigured protocols in SoftMax® Pro Software (see Tables 2 and 3 for instrument-specific settings). Both Microplate Optimization and Read Height adjustment were performed on the SpectraMax i3x and the SpectraMax iD5 readers to ensure optimal assay sensitivity and dynamic range.

Data analysis

Analysis was performed using Cisbio's patented ratiometric reduction method for HTRF assays, based on the two emission wavelengths detected. Analysis steps are shown below. Donor emission at 616 nm is used as an internal reference, while

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.

Parameter	
Optical configuration	HTRF Detection Cartridge
Read mode	TR-FRET
Read type	Endpoint
Wavelengths	Excitation: 340 nm Emission 1: 616 nm Emission 2: 665 nm
PMT and optics	Number of pulses: 30 Excitation time: 0.05 ms Measurement delay: 0.03 ms Integration time: 0.4 ms Read height [optimize]

Table 2. Optimized instrument settings for the HTRF cAMP HiRange assay on the SpectraMax i3x reader.

Parameter	
Read mode	TR-FRET
Read type	Endpoint
Wavelengths	Select 'Use Filter' for both Ex, Em. Excitation: 340 nm Emission 1: 616 nm Emission 2: 665 nm
PMT and optics	Number of pulses: 50 Excitation time: 0.05 ms Measurement delay: 0.1 ms Integration time: 0.6 ms Read height [optimize]

Table 3. Optimized instrument settings for the HTRF cAMP HiRange assay on the SpectraMax iD5 reader. For HTRF reads, the Enhanced TRF module and excitation and emission filters contained in the HTRF Detection System must be installed.

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:

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

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

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

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

(Ratio_{neg} = Ratio of negative control)

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

Results

Data were analyzed as described above and graphed with SoftMax Pro Software using a 4-parameter curve fit (Figure 2). Best results were obtained with the reader settings indicated in Table 2 (SpectraMax i3x reader) or Table 3 (SpectraMax iD5 reader). These settings were optimized in collaboration with Cisbio to certify the readers as HTRF compatible. Changing the delay time, integration time, or number of pulses may lead to less than optimal results. Assay suitability was assessed using the EC_{50} and the signal-to-noise ratio, which should be ≤ 25 nM and ≥ 20, respectively, as indicated by Cisbio. EC_{50} values were very similar for both readers and within acceptable range (Table 4). The signal-to-noise ratio was slightly improved for the SpectraMax iD5 reader, but both readers gave signal-to-noise ratios above the acceptable limits, with CV values below 6%. Both the SpectraMax i3x and the SpectraMax iD5 readers offer high sensitivity and a broad dynamic range for the cAMP G_s HiRange assay.

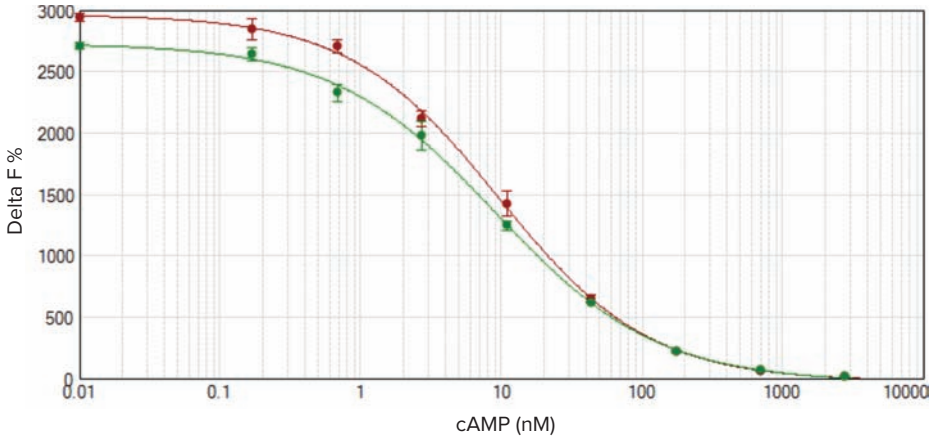

Figure 2. HTRF cAMP calibration curves. HTRF cAMP calibration curves were measured on the SpectraMax i3x (green circles) and SpectraMax iD5 (red circles) readers. The assay window for the SpectraMax iD5 was slightly larger, but the assay quality was excellent for both readers (CV values < 6%, R^2 = 0.999).

Parameter	Passing	SpectraMax i3x	SpectraMax iD5
EC_{50}	≤ 25 nM	9.17	9.66
Signal/noise	≥ 20	28.1	30.5

Table 4. Summary of results for the cAMP HiRange standard curve.

Conclusion

The SpectraMax i3x and SpectraMax iD5 readers can be equipped with an HTRF-certified detection cartridge (i3x) or an HTRF-certified module and filters (iD5) to meet the demanding specifications required for HTRF assays. Both readers demonstrated their ability to perform the cAMP G_s HiRange assay with results well within Cisbio's required range. Data acquisition and analysis are simplified using SoftMax Pro Software with preconfigured HTRF protocols.

Reference

1. http://www.htrf.com/htrf-technology

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