Application Note

HTRF® cAMP/IP-One HTplex™ Cell-Based Assay performed on BioTek's Synergy™ 2 Multi-Mode Microplate Reader

Laurence Jacquemart, Estelle NGarwate, Marie-Laure Lebreton, Cisbio Bioassays
Peter Banks, Xavier Amouretti, BioTek Instruments, Inc.

Multiplexed quantification of cAMP and IP1 accumulation in CHO cells transfected with vasopression receptor, subtype 2, is demonstrated on Synergy™ 2 Multi-Mode Microplate Reader using HTRF® technology.

Introduction

GPCRs carry information within cells via two major signaling pathways: regulation of cAMP levels and increases in intracellular Ca^{2+} triggered by inositol (1, 4, 5) tri-phosphate (IP3). These signaling pathways are activated by the specific G protein associated with the receptor. Gs and Gi coupled receptors result in variations of cAMP while Gq coupled GPCRs activate phospholipase C (PLC) and trigger the inositol phosphate (IP) cascade.

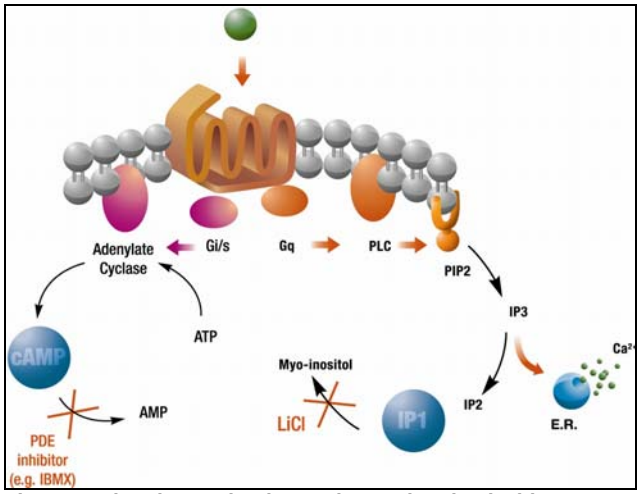


Figure 1. Signal transduction pathways involved with G protein-coupled receptor activation.

Cisbio Bioassays developed a full line of quality, ready-to-use kits capable of monitoring activation of Gs, Gi and Gq coupled receptors and recently introduced a new generation of HTRF technology incorporating Lumi4-Tb™, a new terbium Cryptate.

Thanks to this chemistry, detection of two events at the same time in one well became possible by using 2 different acceptors, a green dye (λ_{Em} = 520 nm) and a red dye (λ_{Em} = 665 nm).

Assay principle

The **HTplex™ cAMP and IP-One assay** is intended for the direct quantitative determination of cAMP and myo-Inositol 1 phosphate (IP1) simultaneously. The assay conditions have been optimized in order to enable cAMP and IP1 assessment in a cell-based assay, using HTRF® technology.

The two molecules are measured using, two monoclonal antibodies labelled with Lumi4-Tb™ as donors (anti-cAMP Cryptate and anti-IP-one Cryptate) and cAMP-green dye and IP-one-red dye as acceptors.

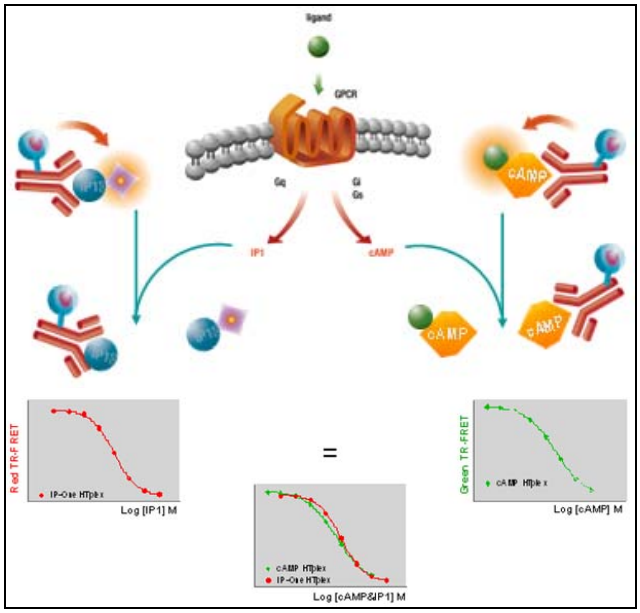


Figure 2. Assay principle is based on competition for limited Ab sites between induced second messenger and fluorescently labeled standard – IP1: red; cAMP: green.

The method is a combination of two competitive immunoassays, the first one between native cAMP produced by cells and cAMP-Green tracer, the second one between native IP1 produced by cells and IP1-Red tracer. The binding is visualized using 2 monoclonal antibodies (respectively MAb anti cAMP and MAb anti IP1) labeled with Lumi4™ –Tb cryptate. The specific emission signals at 520 nm (green dye) and 665 nm (red dye) are inversely proportional to the concentrations of cAMP and IP1 respectively

Materials and Methods

- IP-One and cAMP reagents from Cisbio Bioassays
- White 384 microplate, Greiner Bio One
- Reader: Synergy™ 2 from BioTek Instruments, Inc.

Day1, cells are plated in ~~the~~ white microplate and incubate overnight at 37°C in CO_2 atmosphere. On day 2 cell supernatant is removed and replaced by 10 µL of stimulation buffer. Dilutions of vasopressin prepared in stimulation buffer are added (10 µL) and after 40 minutes of stimulation at 37°C, 5 µL of a mixture of the two acceptors (cAMP-green dye +IP1-red dye) and 5 µL of the mixture of the two specific MAbs labeled with Lumi4™-Tb are dispensed. For calibration curves~~,~~ 20 µL of cAMP/IP1 mix calibrator is dispensed prior to the acceptor and donor HTRF® conjugates.

The microplate is then incubated for 1 hour at room temperature and afterwards time-resolved fluorescence is measured on the Synergy 2.

Data reduction

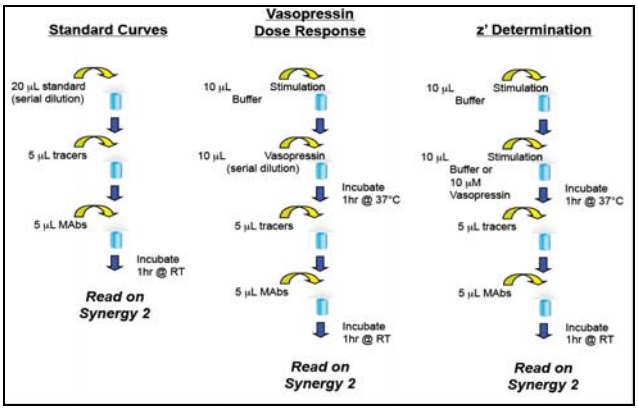
Cisbio has developed and patented a ratiometric measurement that uses both the emission wavelength of the donor and acceptor (patent US 5,527,684 and foreign equivalents) to correct for well-to well variability and signal quenching from assay components and media. Emissions at 620 nm (donor) are used as an internal reference while emissions at 665 nm and 520 nm (acceptor) are used as an indicator of the biological reaction being assessed.

Three sequential measurements are carried out: 620 nm for the Cryptate emission, 520 nm and 665 nm for specific signals emitted by the two acceptors: green and red dyes. Ratios of fluorescence intensities 665/620 and 520/620 (acceptor/donor) are calculated in order to detect each single interaction.

1. Reagents and reconstitution

	Reagents	Working solutions
Frozen set	Anti-cAMP-Tb Cryptate	Dilute first each conjugate 30 fold in conjugate & lysis buffer then mix vol/vol (1 vol. of anti-IP1-Tb working solution to 1 vol. of anti-cAMP-Tb working Solution). In order to obtain the "Donor mix" solution.
	Anti-IP1-Tb Cryptate	
	cAMP-Green	Dilute first each conjugate 30 fold in conjugate & lysis buffer then mix vol/vol (1 vol. of anti-IP1-Tb working solution to 1 vol. of anti-cAMP-Tb working Solution). In order to obtain the "Acceptor mix" solution.
	IP1-Red	
	cAMP/IP1 mix standard	See calibrator curve preparation in the table below
Buffer set	Stimulation buffer 5X	Make a 1/5 dilution in distilled water to prepare the 1X stimulation buffer. Add a phosphodiesterase inhibitor such as IBMX in order to prevent cAMP degradation (e.g. 1mM IBMX).
	Conjugate & lysis buffer	Ready to use

2. Assay protocol



3. Calibration curve

HTplexTM calibrator	Preparation	cAMP working solution (nM)	IP1 working solution (nM)
Calibrator 8	50 µL cAMP/IP1 mix standard + 450 µL stimulation buffer 1X	4200	16500
Calibrator 7	100 µL Cal.8 + 300 µL of stimulation buffer 1X	1050	4125
Calibrator 6	100 µL Cal.7 + 300 µL of stimulation buffer 1X	262,5	1031
Calibrator 5	100 µL Cal.6 + 300 µL of stimulation buffer 1X	65,6	258
Calibrator 4	100 µL Cal.5 + 300 µL of stimulation buffer 1X	16,4	64,5
Calibrator 3	100 µL Cal.4 + 300 µL of stimulation buffer 1X	4,1	16,1
Calibrator 2	100 µL Cal.3 + 300 µL of stimulation buffer 1X	1	4
Calibrator 1	100 µL Cal.2 + 300 µL of stimulation buffer 1X	0,3	1
Calibrator 0	Stimulation buffer 1X	0	0

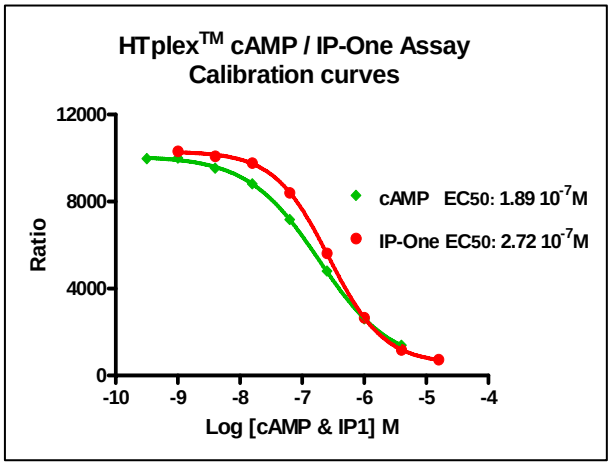


Figure 3. Calibration curves.

4. Cells preparation

CHO-V2R cells (stable transfection with the vasopressin-receptor gene) are cultivated in F12 medium. After counting, cells are diluted in order to obtain cell density of 1,000,000 cells/mL, and then 30 µL are dispensed in each well (30,000 cells/well). The plate is incubated for an over night incubation at 37°C. Then cell supernatant is aspirated and immediately replaced with 10 µL of stimulation buffer.

5. Vasopressin dose-response curve

Vasopressin from 2.10-4 M to 1.5. 10-4 M (working solutions) in stimulation buffer 1X with a six fold dilution series between each concentration.

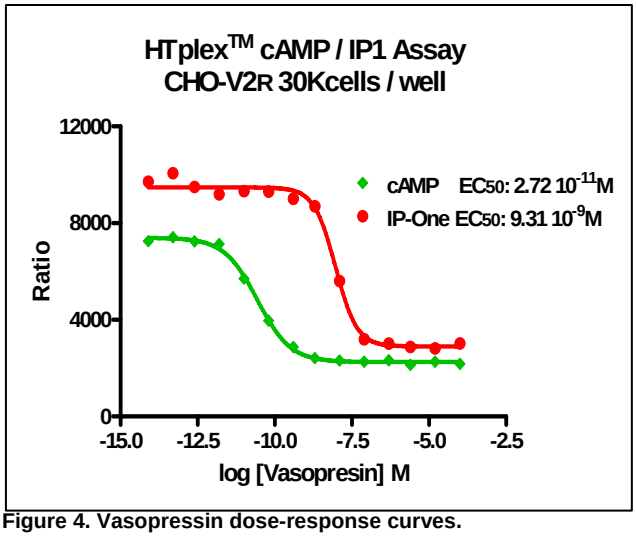


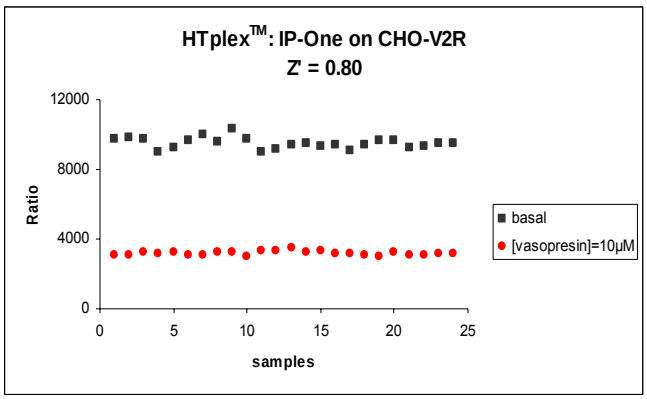
Figure 4. Vasopressin dose-response curves.

EC50 obtained for cAMP is 2.7 10-11M and 9.3 10-9 M for IP1. The windows were similar for the 2 analytes = 3.3

6. Screening robustness – Z' factor calculation

Twenty-four replicates were run using Vasopressin 10 µM (EC100) and 0 µM (basal).

Z' factor was calculated between stimulated and non stimulated cells.



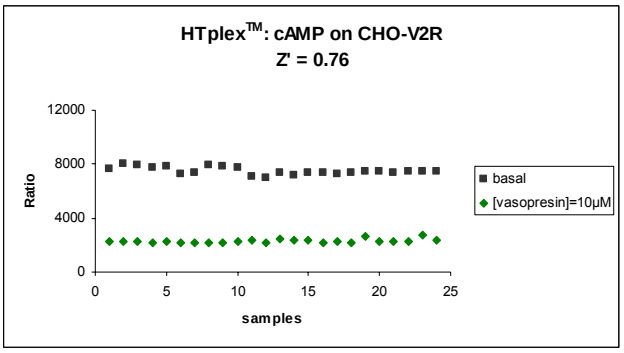


Figure 5. Z' factor calculation.

The above results show the excellent assay robustness (Z≈ 0.8) for both assays.

7. Synergy™ 2 Settings

Synergy 2 readers must be appropriately configured for HTRF® Tb readout by setting up the measurement conditions in the Gen5™ Data Analysis Software. In particular, these parameters should be entered as defined in the table below. The Synergy 2 must be equipped with the TRF module.

Three sequential measurements should be carried out: 620 nm for the cryptate emission, 520 nm and 665 nm for specific signals emitted by the two acceptors: green and red dyes. Ratios* of fluorescence intensities 665/620 and 520/620 (acceptor/donor) must be calculated in order to detect each single interaction.

	Read 1	Read 2	Read 3
Emission filter	**620/10**	**665/8**	**520/10**
Excitation filter	**340/30**		
Optics Position	**Top 400 nm**		
Number of flashes	**10**		
Lag time	**100 µs**		
Integration time	**300 µs**		
Sensitivity	**Optimise on highest signal**		
Z height	**Default**		

Discussion

The determination of both cAMP and IP1 accumulation in the same microplate is demonstrated in both calibration curves (Figure 3) and vasopressin dose-response curves (Figure 4). Using the standard curve to transform ratio signals to pmol amounts of second messenger, EC_{100} amounts of vasopressin induce 43±5 pmol of cAMP and 26±2 pmol of IP1 from the activation of vasopressin receptor R2. There is almost a 3 orders of magnitude difference in EC50 for vasopressin for cAMP generation relative to IP1 generation.

Rev. 6/16/09