GPCR Activation is Measured Using Cisbio's cAMP and IP1 HTRF® HTplex™ Cell-based Assay

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- Simultaneously measure Gαi/s and Gαq signaling in the same assay upon activation of any G protein-coupled receptor (GPCR)
- New Lumi4-Tb™ HTRF® technology is used to measure both cAMP via a green readout (520 nm) and IP1 via a red readout (665 nm)
- Activation of the vasopressin-2 receptor was measured in CHO cells using the PHERAstar and SDE detection

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- triphosphate (IP3). These signaling pathways are activated by the specific G protein associated with the receptor[1]. Activation of a Gs or Gi coupled receptor results in the increase or decrease of cAMP levels, respectively. While activation of a Gq coupled receptor activates phospholipase C (PLC) and triggers the inositol phosphate (IP) cascade (Figure 1).

Cisbio Bioassays has developed assay kits capable of monitoring the activation of Gs, Gi and Gq coupled receptors, using their new generation Lumi4-Tb™ TR-FRET Cryptate. This chemistry allows for the detection of two events in one well using two different acceptors, a green dye (λ = 520 nm) and a red dye (λ = 665 nm). This same chemistry is used in Cisbio's Tag-lite® technology.

IP-One and cAMP experiments were performed on the PHERAstar *Plus* HTS microplate reader using Simultaneous Dual Emission detection. With this unique feature, the plate is read only once for dual emission assays, thereby decreasing time and variability. All Cisbio chemistry can be performed on the PHERAstar *FS*, which uses a UV Laser for excitation. The UV Laser will allow for greater sensitivity and faster read times. The POLARstar Omega and FLUOstar Omega microplate readers with advanced assay technology also perform this chemistry, but in a non-HTS fashion.

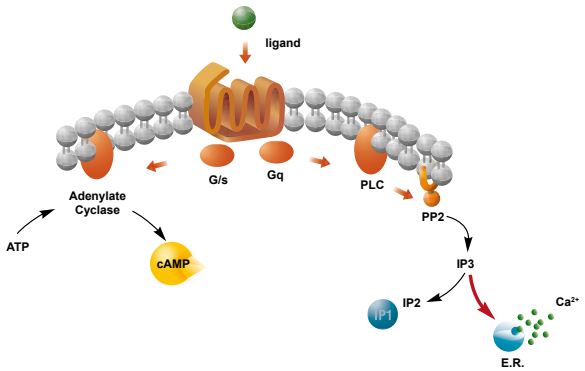


Fig. 1: *The HTplex™ Assay from Cisbio measures GPCR activation via two second messenger responses, cAMP and IP1, in one experiment.*

Assay Principle

The IP-One and cAMP HTplex™ assay is a competitive immunoassay that uses two antibodies labelled with Lumi4-Tb™ (anti-cAMP Cryptate and anti-IP1 Cryptate as donors) and two acceptors (cAMP-green dye and IP1-red dye). In the inactivate state, a high TR-FRET signal is seen for both the red and green emissions. As cAMP or IP1 is produced upon GPCR activation, the tracer green-cAMP or the tracer red-IP1 will be uncoupled from the Tb-cryptate antibody, thereby causing a decrease in TR-FRET signal. Specific emission signals are inversely proportional to the concentration of cAMP and IP1 in a standard or in a cell lysate.

Materials and Methods

- ☐ IP-One and cAMP reagents from Cisbio Bioassays, France
- ☐ White 384-well format microplate, Greiner, Germany
- ☐ PHERAstar *Plus* multidetection microplate reader from BMG LABTECH, Germany

Cells preparation

CHO-V2R cells (stable transfection with the vasopressin-receptor) are cultivated in F12 medium then diluted to obtain a concentration of 1,000,000 cells/mL (viability: 96.3%). Then 30 µL are distributed in each well (giving 30,000 cells/well). The plate is incubated at 37°C overnight. The cell supernatant is aspirated (the cells collapse to the well bottom) and immediately replaced with 10 µL of stimulation buffer.

For standard curve: add 20 µL of diluted standards, 5 µL cAMP-green dye/IP1-red dye and 5 µL cAMP-Cryptate/ IP1-Cryptate, then incubate for 1 hr at RT. For vasopressin dose-response: add 10 µL stimulation buffer, 10 µL vasopressin (14 dilutions: from 0 to 10^{-4} M), 1 hr stimulation at 37°C. Then add 5 µL cAMP-green dye/IP1-red dye, 5 µL cAMP-Cryptate/ IP1-Cryptate and incubate for 1 hr. For Z' calculation: add 10 µL stimulation buffer (basal) or 10 µL vasopressin 10 µM, 1 hr stimulation at 37°C. Then add 5 µL cAMP-green dye/IP1-red dye, 5 µL cAMP-Cryptate/IP1-Cryptate and incubate for 1 hr.

PHERAstar Set-Up

Optic modules: two HTplex™ modules, one for red (337/665/620) and one for green (337/620/520).
Integration start = 60 µs, integration time = 400 µs.
Flash number = 300 (if speed is important, 100 flashes can be used).
Plate is read twice using each module (set multichromatics to 2).

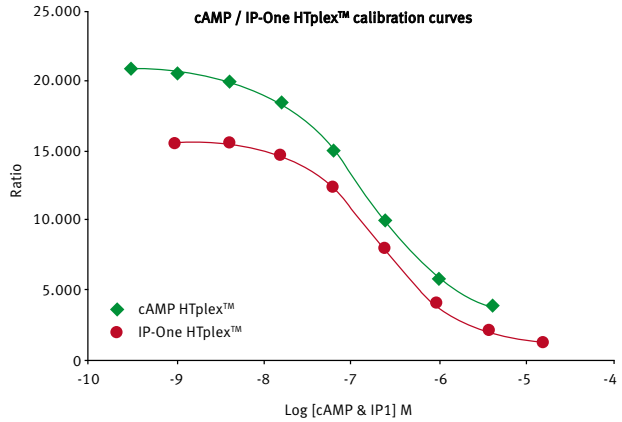
For UV Laser on PHERAstar *FS*, it is recommended to use 7 flashes. For FLUOstar Omega or POLARstar Omega with Advanced Assay Technology, use a constant gain of 2500 for both channels, 200 flashes and read the plate 3 times using multichromatics (665, 620, and 520).

Data Reduction

Cisbio has a patented 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. 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. Ratios of fluorescence intensities 665/620 and 520/620 (acceptor/donor) are calculated in order to detect each single interaction.

Results and Discussion

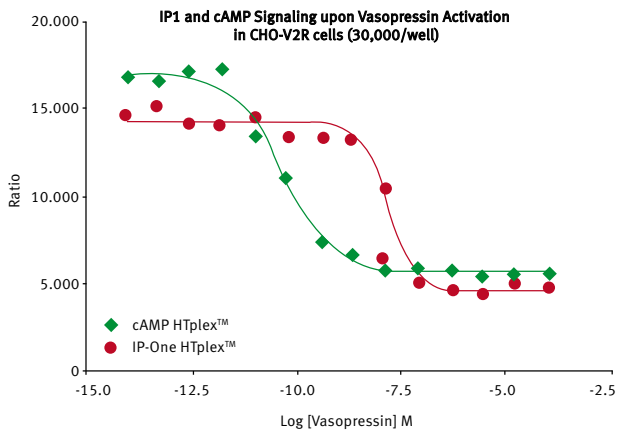
Figure 2 shows the calibration curve using standards for cAMP and IP1. Both curves give the expected EC_{50} values.



	cAMP HTplex™	IP-One HTplex™
BOTTOM	2820	1165
TOP	20901	15812
EC_{50}	**1.51E-07**	**2.28E-07**

Fig. 2: *Calibration curves for the HTplex™ assay using standards for cAMP (green) and IP-One (red).*

This figure shows the occurrence of two signaling events - an initial cAMP (Gs) response at lower concentrations of vasopressin presumably through activation of endogenous V1 receptors; and a delayed IP3 (Gq) response at higher concentrations through activation of the transfected V2 receptor (as measured by IP1 accumulation). EC_{50} values and assay windows are shown.



	Vasopressin dose response	
	cAMP HTplex™	**IP1 HTplex™**
BOTTOM	5552	2699
TOP	17058	9040
WINDOW	3.1	3.3
EC_{50}	**4.61E-11**	**15.6E-09**

Fig. 3: *Using the HTplex™ assay and PHERAstar Plus, GPCR signaling in CHO-V2R cells was measured via cAMP (green) and IP1 (red) responses upon a dose dependent activation of vasopressin.*

In Figure 3, both cAMP and IP1 were measured in CHO-V2R cells upon activation with vasopressin in a dose response manner.

Lastly, the robustness of the assay as measured by the Z prime calculation (Figure 4) shows that both signals are well above the reliability range for an HTS assay, giving Z' values › 0.75.

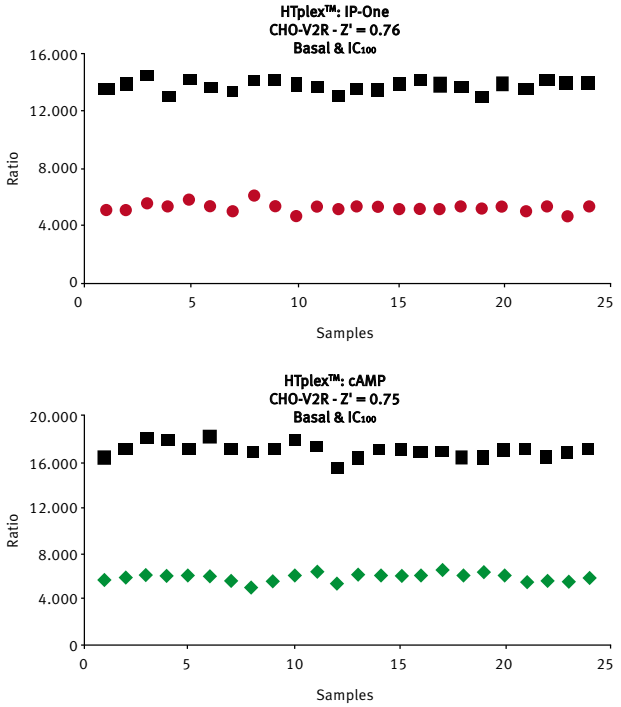


Fig. 4: *Z prime calculations for IP1 (top graph, red) and cAMP (bottom graph, green) using 0 and 10 µM of vasopressin.*

Conclusion

The HTplex™ assay from Cisbio can evaluate two different GPCR signaling pathways, Gαi/s and Gαq, through the measurement of their second messenger responses, cAMP and IP3 (via IP1), respectively. Herein, the HTplex™ assay was used to evaluate the dose response effect of vasopressin on CHO-V2R cells. As measured using the PHERAstar *Plus* with Simultaneous Dual Emission detection, there is an initial cAMP response at lower concentrations and a latent IP1 accumulation at higher concentrations. The concept of this application note can be extended to Cisbio's complimentary HTRF® chemistry, Tag-lite®, which uses the same Lumi4™-Tb cryptate and the same red and green acceptors.

References

1. Woehler, A., Ponimaskin, E.G. (2009) *Curr. Mol. Pharmacol.*; **2(3)**: 237-248.

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