cAMP / IP-One HTplex cell-based experiment performed on Mithras LB 940 using HTRF® technology

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Introduction

GPCRs carry information within cells via two major signaling pathways: regulation of cAMP levels and increases in intracellular Ca2+ 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.

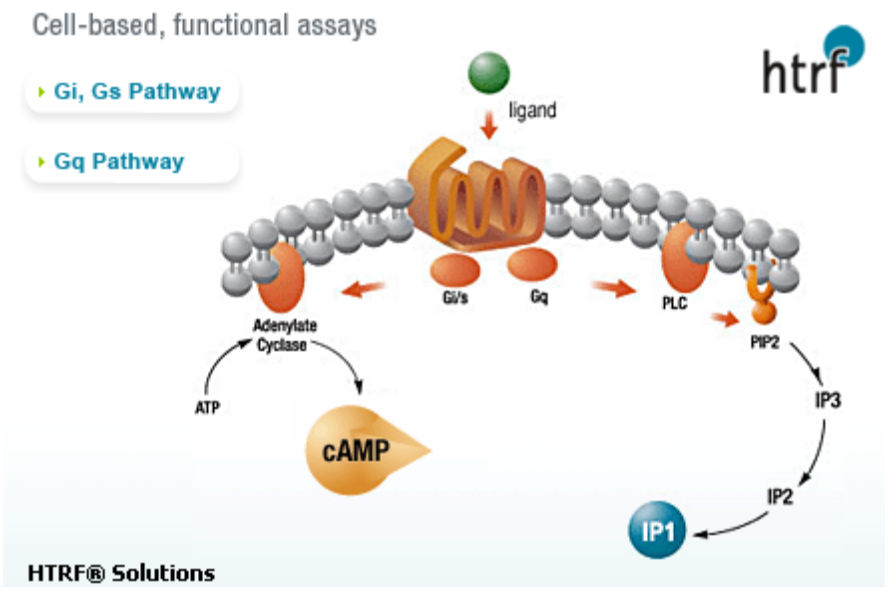

Figure 1. GPCR signalling pathways.

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 TR-FRET terbium Cryptate.

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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 (cwl = 520 nm) and a red dye (cwl = 665 nm)

Assay principle

The IP-One and cAMP HTplexTM assay is competitive immunoassay that uses Terbium cryptate-labeled anti-IP1 MAb, cryptate-labeled anti-cAMP MAb, red dye-labeled IP1 and green dye-labeled cAMP.

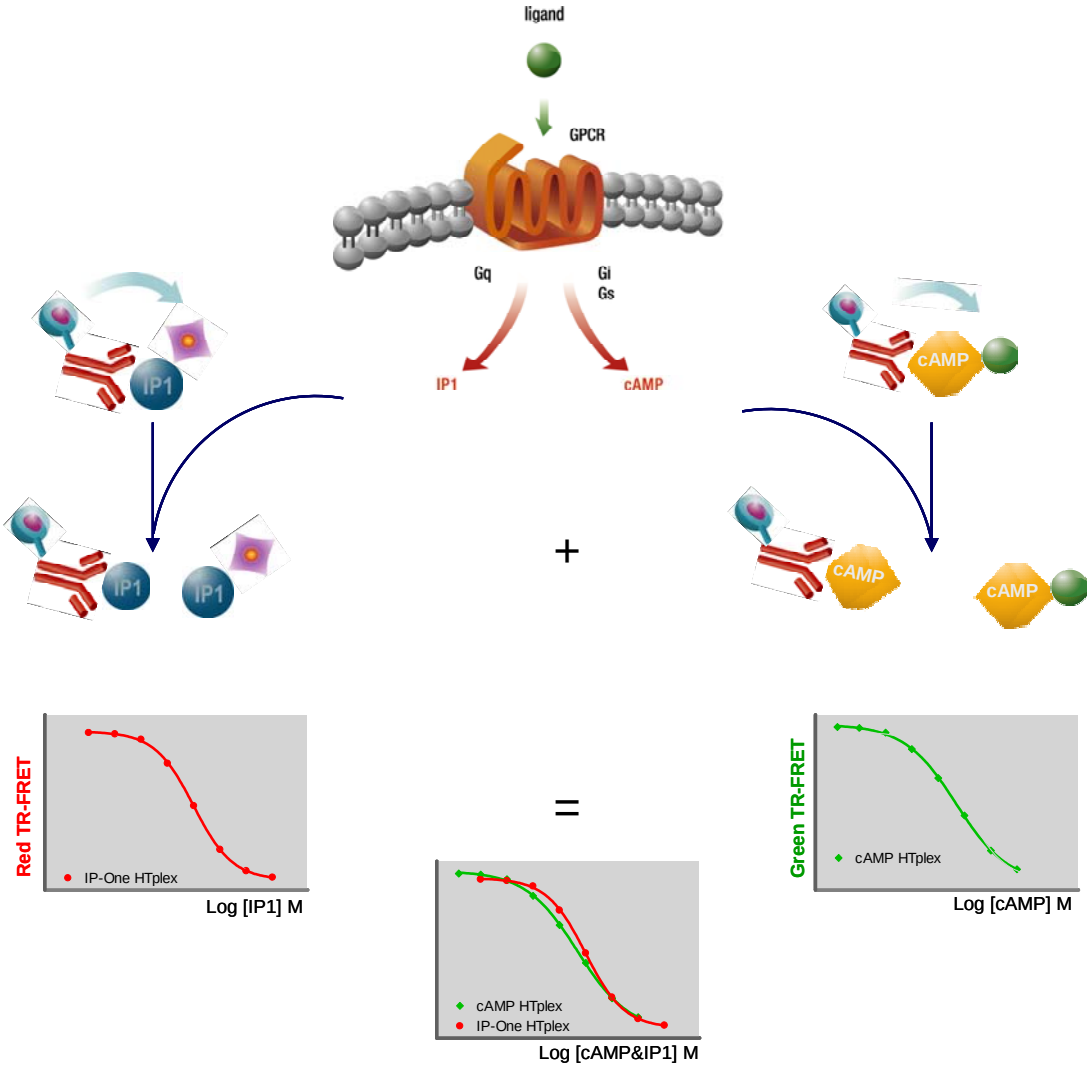

Figure 2. HTplex assay principle.

The two molecules are measured in a format assay using two antibodies labelled with Lumi4-TbTM (anti-cAMP Cryptate and anti-IP-one Cryptate as donors) and two acceptors (cAMP-green dye and IP-one-red dye). The two donors are mixed together and the two acceptors are also mixed.

The assay is based on the competition of the native IP1 produced by cells and IP1 coupled to the red dye towards a monoclonal antibody specific for IP1, labeled with the Lumi4™-Tb cryptate coupled to the competition of the native cAMP produced by cells and cAMP coupled to the green dye towards a monoclonal antibody specific for cAMP, labeled with the Lumi4™-Tb.

Specific emission signals are inversely proportional to the concentration of cAMP and IP1 in a standard or in a cell lysate.

Mithras LB 940

The Mithras LB 940 is a multimode plate reader with a unique optical design (DOPS – Dedicated Optical Path System) to ensure optimized performance for the detection technologies implied. These are HTRF®, TRF, luminescence, BRET/BRET², fluorescence, absorbance, fluorescence polarization and AlphaScreen™. In the Mithras each technology has its own dedicated optical system meeting the specific requirements, e.g. cross talk elimination in luminescence. In addition accessory options like reagent injectors, temperature control and cooled PMT detection units are available. The fact that injectors are located in the reading position fast reaction kinetics can be monitored.

Figure 3. Mithras LB 940 multimode reader for microplates.

For the HTplex assays the Mithras HTRF® model can be equipped with an additional emission filter (cwl 520 nm) for monitoring the specific emission of the green acceptor besides the standard emission wavelengths of 620 nm and 665 nm used in HTRF®.

Assay protocol

On day 1 cells are plated in white plates and incubated overnight at 37 °C in CO_2 atmosphere. On day 2 the 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 time of stimulation at 37 °C, 5 µL of a mixture of cAMP-red dye and IP1-green dye and 5 µL of the mixture of the 2 specific MAb labeled with Lumi4™-Tb are dispensed. For calibration curves 20 µL of calibrators containing a mixture of cAMP and IP1 is dispensed prior to the acceptor and donor HTRF® conjugates.
The plate is incubated for 1 h at room temperature and afterwards time-resolved fluorescence is measured on the Mithras using the HTRF® reading module.

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 520nm (acceptor) are used as an indicator of the biological reaction being assessed.

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

Ratio (red emission) = (signal @ 665 nm / signal @ 620nm) x 10^4

Ratio (green emission) = (signal @ 520 nm / signal @ 620nm) x 10^4

Z' = $1 - [(3 * \text{STANDARD DEV}_{\text{RATIO MAX}} + 3 * \text{STANDARD DEV}_{\text{RATIO MIN}}) / |\text{RATIO}_{\text{MAX}} - \text{RATIO}_{\text{MIN}}|]$

Reagents and reconstitution

HTplex reagents

HTplex	HTplex conjugate & lysis buffer	Ready to use
	HTplex stimulation buffer 5x + IBMX	1 vol stim. buffer 5x + 4 vol H_2O + IBMX 1mM
	IP1-red dye/cAMP-green dye	After dilution 1/30 of each conjugate, mix them v/v
	Anti-IP1 Cryptate Anti-cAMP Cryptate conjugates	After dilution 1/30 of each conjugate, mix them v/v
	cAMP/IP1 mix calibrator	

Table 1. HTplex™ reagents.

HTplex calibrator	cAMP working solution (nM)	IP1 working solution (nM)	Preparation
Standard 8	4200	16500	40 µL stock sol + 360 µL stim buffer
Standard 7	1050	4125	100 µL std8 + 300 µL of stim buffer
Standard 6	262.5	1031	100 µL std7 + 300 µL of stim buffer
Standard 5	65.6	258	100 µL std6 + 300 µL of stim buffer
Standard 4	16.4	64.5	100 µL std5 + 300 µL of stim buffer
Standard 3	4.1	16.1	100 µL std4 + 300 µL of stim buffer
Standard 2	1	4	100 µL std3 + 300 µL of stim buffer
Standard 1	0.3	1	100 µL std2 + 300 µL of stim buffer
Standard 0	0	0	Stim buffer

Table 2. HTplex™ calibrator dilutions.

Vasopressin dose-response curve

Dilution	**[vasopressin] M (Stimulation step)**	**[vasopressin] M Initial (Working step)**	**Preparation**
14	1.0E-04	2.0E-04	60 µL stock solution + 540 µL stim buffer
13	1.7E-05	3.3E-05	100 µL dilution 14 + 500 µL stim buffer
12	2.8E-06	5.6E-06	100 µL dilution 13 + 500 µL stim buffer
11	4.6E-07	9.3E-07	100 µL dilution 12 + 500 µL stim buffer
10	7.7E-08	1.5E-07	100 µL dilution 11 + 500 µL stim buffer
9	1.3E-08	2.6E-08	100 µL dilution 10 + 500 µL stim buffer
8	2.1E-09	4.3E-09	100 µL dilution 9 + 500 µL stim buffer
7	3.6E-10	7.1E-10	100 µL dilution 8 + 500 µL stim buffer
6	6.0E-11	1.2E-10	100 µL dilution 7 + 500 µL stim buffer
5	9.9E-12	2.0E-11	100 µL dilution 6 + 500 µL stim buffer
4	1.7E-12	3.3E-12	100 µL dilution 5 + 500 µL stim buffer
3	2.8E-13	5.5E-13	100 µL dilution 4 + 500 µL stim buffer
2	4.6E-14	9.2E-14	100 µL dilution 3 + 500 µL stim buffer
1	7.7E-15	1.5E-14	100 µL dilution 2 + 500 µL stim buffer

Table 3. Vasopressin dilutions.

Cells preparation

CHO-V2R cells (stable transfection with the vasopressin-receptor gene) are cultivated in F12 medium. After counting the cells (almost 3 000 000 cells/mL; viability: 96.3 %), the culture media is diluted to obtain a concentration of 1 000 000 cells/mL. Then 30 µL are

distributed in each well (so 30 000 cells in each well). The plate is incubated at 37 °C overnight.

After that, the cell supernatant is aspirated (the cells collapse on the bottom of the wells) and immediately replaced with 10 µL of stimulation buffer.

Assay protocols

Standard curve

20 µL standard

5 µL cAMP-green dye/IP1-red dye

5 µL anti-cAMP-Cryptate/anti-IP1-Cryptate

1 h incubation at room temperature

Vasopressin dose-response

10 µL stimulation buffer

10 µL vasopressin (14 dilutions: from 0 to 10^{-4} M)

1 h stimulation at 37 °C

5 µL cAMP-green dye/IP1-red dye

5 µL anti-cAMP-Cryptate/anti-IP1-Cryptate

1 h incubation at room temperature

Z' calculation

10 µL stimulation buffer

10 µL stimulation buffer (basal) or 10 µL vasopressin 10 µM

1 h stimulation at 37 °C

5 µL cAMP-green dye/IP1-red dye

5 µL anti-cAMP-Cryptate/anti-IP1-Cryptate

1 h incubation at room temperature

Instrument Settings

The Mithras LB 940 reader must be equipped with the TR-FRET reading module which includes the necessary optical components for HTRF® readout. Instruments equipped with the "Flash Filter Wheel" option are recommended as a specific excitation filter optimized for Tb cryptates can be used together with an optimized excitation filter for Eu cryptates.

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

The Mithras LB 940 operating software comes with preset ready-to-use parameter files for HTRF® measurements including the ratio calculation. The recommended settings are defined under the TR-Fluorescence protocol as described below.

	Measurement 1	Measurement 2	Measurement 3
Excitation filter!	TRF340 (26) (by default)	TRF340 (26) (by default)	TRF340 (26) (by default)
Emission filter	**D620 (TRF)**	**D665 (TRF)**	**D520 (TRF)**
Lamp Energy	100	100	100
Cycle time	2000 µs	2000 µs	2000 µs
Delay time	50 µs	50 µs	50 µs
Reading time	300 µs	300 µs	300 µs
Counting time	1 s Optimal	1 s Optimal	1 s Optimal
Operation mode	by plate	by plate	by plate

Table 4. Instrument settings for HTplex™.

* The fluorescence ratio is a correction method developed by Cisbio international with an application limited to the use of HTRF® reagents and technology, and for which Cisbio international has granted a licence to Berthold Technologies. The method is covered by the US patent 5,527,684 and its foreign equivalents.

! D320 (standard TRF excitation filter for Eu cryptates) may be used for white microplates only!

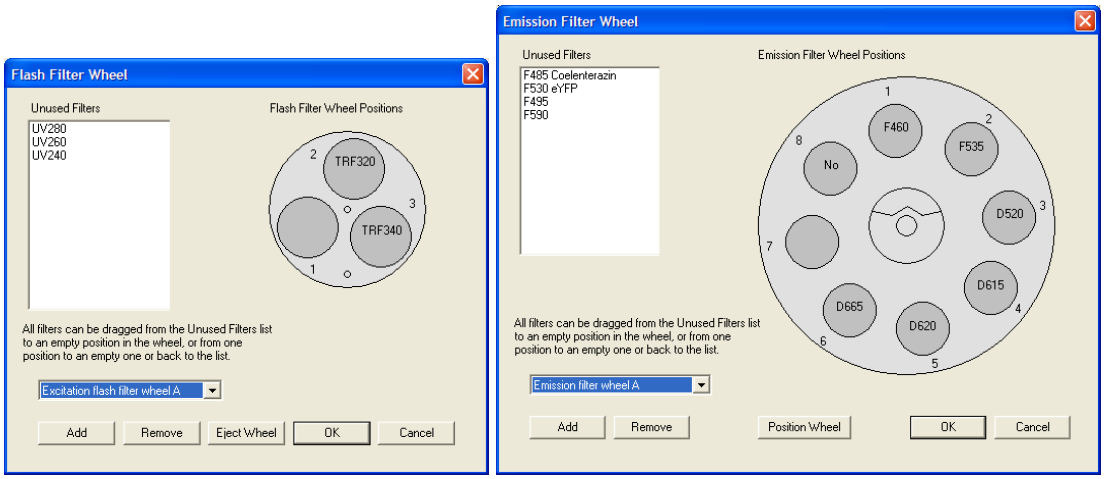

Figure 4a. Instrument settings for HTplex™. Global definition of the optimum excitation filter ***TRF340*** for Tb cryptates and the emission filters ***D520***, ***D620*** and ***D665*** for the specific emission spectra of donor and acceptors.

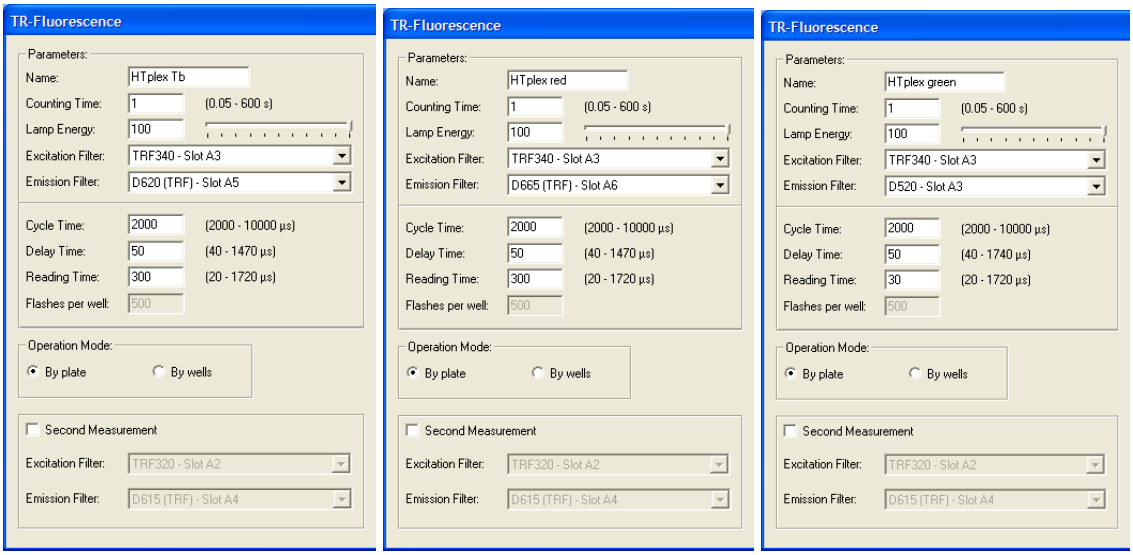

Figure 4b. Instrument settings for HTplex™. ***Operations*** dialogues with filter and timing definitions for the 3 sequential measurements of donor, red acceptor and green acceptor.

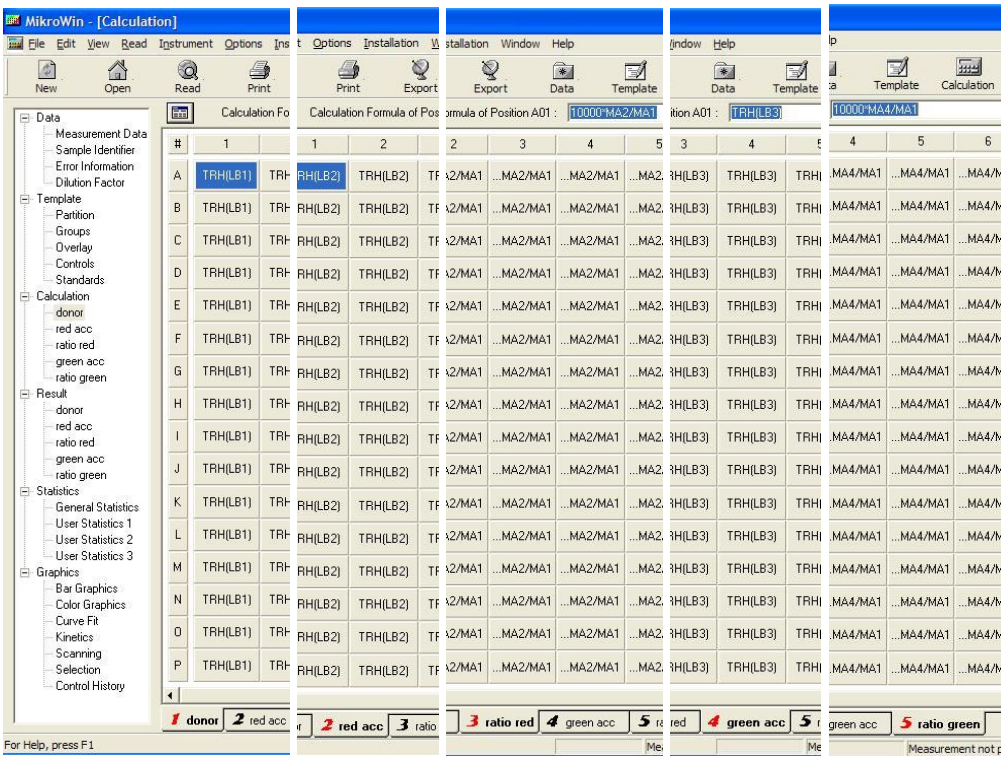

Figure 4c. Instrument settings for HTplex™. ***Calculation Matrices*** holding the individual readings and the calculations of the red and the green ratios for each well. Each of the matrices can be selected for data export into spreadsheet or data base software.

Results

Calibration curves

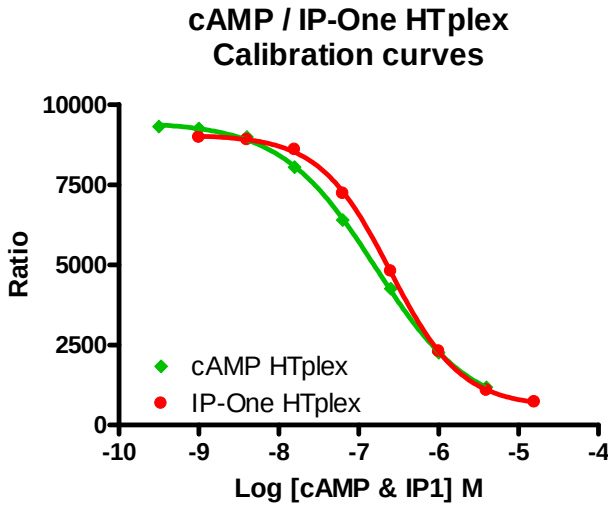

Figure 5. cAMP (green diamonds) and IP1 (red dots) calibration curves.

	cAMP HTplex	IP-One HTplex
BOTTOM	385	573
TOP	9459	9053
EC50	**1.63E-07**	**2.48E-07**

Table 5. Statistics for cAMP and IP1 calibration curves.

Figure 5 and Table 5 show an assay window of >9000 for the HTplex cAMP and ~8500 for HTplex IP1. The EC50 values can be determined as 163 nM and 248 nM respectively.

Vasopressin dose-response

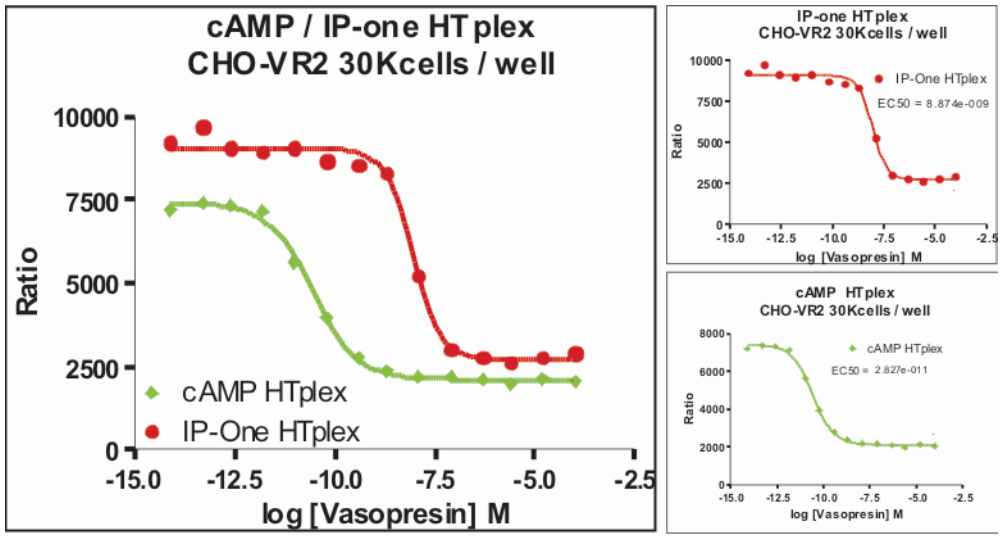

Figure 6. Vasopressin dose-response monitored with cAMP (green diamonds) and IP1 (red dots) production.

	cAMP HTplex	IP-One HTplex
BOTTOM	2090	2699
TOP	7399	9040
S/N	3.5	3.3
EC50	**2.83E-11**	**8.87E-09**

Table 6.. Statistics for Vasopressin dose-response curves (HTplex cAMP and IP1).

The Vasopressin dose-response experiments yielded EC50 values of 28.3 pM and 8.87 nM for cAMP and IP1 respectively.

Z' calculation

The HTplex cAMP assay has a Z´ of 0.72 whereas the HTplex IP1 assay has a Z´ of 0.75.

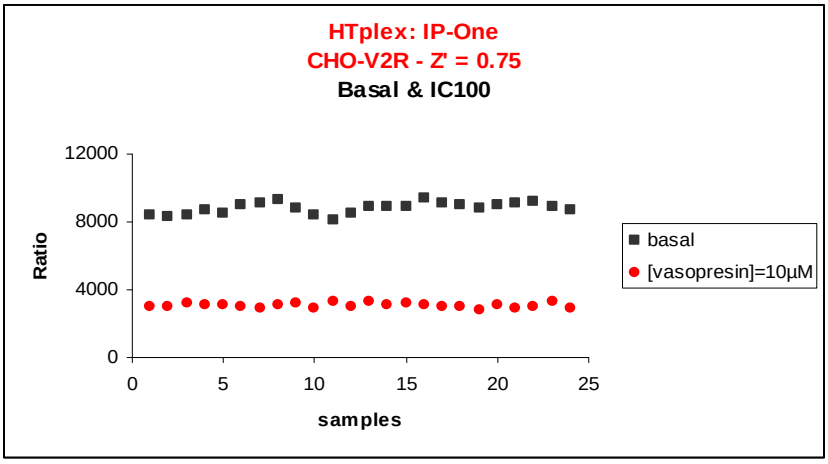

Figure 7. Z´ of HTplex IP1 assay: basal level (black squares) and Vasopressin 10 µM (red dots).

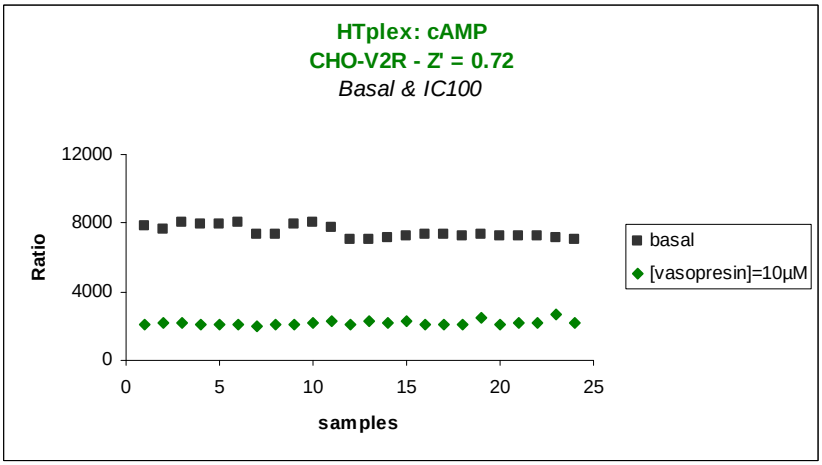

Figure 8. Z´ of HTplex cAMP assay: basal level (black squares) and Vasopressin 10 µM (green diamonds).

Conclusion

The HTplex™ assay for cAMP and IP1 yields excellent Z´ values of 0.72 and 0.75 respectively, which are suitable for screening applications.

The Vasopressin EC_{50} values of both IP1 HTplex™ and the cAMP HTplex™ are perfactly matching those obtained with the single assys:

IP1 HTplex™	$8.87 * 10^{-9}$ M	IP1 HTRF®	$10.4 * 10^{-9}$ M
cAMP HTplex™	$2.83 * 10^{-11}$ M	IP1 HTRF®	$0.87 * 10^{-11}$ M

Also the assay windows for both analytes are comparable to the assay windows of the single assays (data not shown).

The 520 nm emission filter selected yielded excellent results with the Mithras LB 940 multimode reader and will be chosen as the filter of choice for the green acceptor´s emission measurement.

Materials

- IP-One and cAMP reagents (Cisbio Bioassays)
- White 384 microplate (Greiner)
- Mithras LB 940 multimode reader HTRF model (Berthold Technologies)
- Filters (Berthold Technologies):
  excitation TRF 340 nm (ID No. 54083)
  emission 520 nm (ID No. 38836)

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