

HTRF® IP-One assay performed on the PHERAstar® FS microplate reader

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- HTRF® IP-One assay for Gq pathway investigation under HTS conditions
- Excellent performance of the PHERAstar® FS in terms of EC_{50} and Z' (>0.78)
- Comprehensive list of GPCRs already validated with IP-One assay for agonist responses

Introduction

HTRF® (homogeneous time-resolved fluorescence) technology, developed by Cisbio international, is used in assay development and drug screening. HTRF® is based on FRET between a Eu^{3+} cryptate (donor) and a second fluorescent label (acceptor). A new acceptor, d2, allows the introduction of a complete GPCR (G protein-coupled receptor) platform suitable for drug discovery. Upon activation, GPCRs carry the information within the cell via two major signalling pathways: the activation of $G_{\alpha s}$ or $G_{\alpha i}$ coupled GPCRs results in a variation of the cAMP level, whereas the activation of Gq coupled GPCRs result in a transient increase of intracellular Ca^{2+} triggered by inositol [1,4,5] triphosphate (IP3). Cyclic AMP and IP3 therefore represent two essential secondary messengers for monitoring the activity of most GPCRs.

Concerning the Gq pathway, the precursor molecule for the signaling cascade, IP3, is an extremely instable product (turnover only a few tens of seconds) and its degradation is irreversible. IP3 induces a transient calcium release in the cell. Calcium sensing, through a very remote indicator, can allow the high throughput investigation of GPCR activity. To date, there has been no widespread use of IP assays in HTS, given its extremely challenging implementation. For lack of better options, the reference method consists of an assay which detects the accumulation of the cascade's different inositol phosphates (IP3, IP4, IP2 and IP1) after the radioactive precursor's uptake and a separation by affinity.

Recently Cisbio launched a major new kit, HTRF® IP-One, for Gq pathway investigation under HTS conditions. The IP-One kit allows the quantification of the cellular accumulation of inositol 1 phosphate (IP1). This application note focuses on the IP1 assay which has been validated on a broad and representative selection of Gq coupled receptors using the multimode plate reader PHERAstar® FS from BMG LABTECH. This HTRF® certified compatible reader is capable to simultaneously detect the fluorescence at two wavelengths (620 nm and 665 nm) and further signal ratioing also enables the technology to overcome interference from the medium or from compounds.

Assay Principle

The final stage of the cascade when IP1 is transformed into myo-inositol can be blocked with the use of LiCl, which enables one of the Gq pathway's essential sub-products to be stabilized (figure 1), just as IBMX action prevents

cAMP degradation. In its final configuration, it brings into play a highly specific MAb coupled to europium cryptate and the IP1 conjugated to the new HTRF® acceptor, d2. This tiny proprietary molecule, with photophysical characteristics similar to those of XL665 (the reference HTRF® acceptor), improves the technology's performances markedly, particularly in terms of IC_{50} stability and of measurement dynamics.

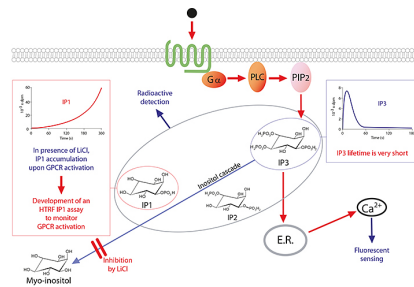
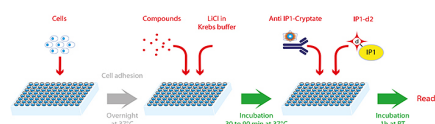


Fig. 1: Gq coupled receptor activation induces IP3 release catalysed by PLC. IP3 degradation occurs rapidly and leads ultimately to the production of IP1. Processing of IP1 into myo-inositol can be prevented by the addition of LiCl.

Materials & Methods

Cisbio's homogeneous IP-One assay can be carried out in a single microplate, into which the cells have been dispensed the day before the actual test is run. The cell stimulation conditions meet the particular characteristics of the cell line used - generally 30 minutes at 37°C. Quantification of the accumulated IP1 is obtained after dispensing the two diluted conjugates into the lysis solution. Measurements can be taken after just one hour of incubation, and be repeated as many times as necessary without impacting final data (e.g. IC_{50}). The standard curve for the kit was run according to the package insert protocol in white 384-well plates (COSTAR cat# 3711384) with 20 μ L total assay volume. The HTRF® signal was read on the PHERAstar® FS.



Cells were kindly provided by the Institut de Genomique Fonctionnelle (IGF), Montpellier, France and Euroscreen, Gosselie, Belgium.

Cell lines (see table 1) expressing the GPCR target



of interest were used for measuring IP1 production by stimulation of the ligand. In a 384-well format, the cell suspension was dispensed at 15,000 cells/20 μ L/well. After incubation at 37°C, the culture supernatants were completely discarded. Then 10 μ L of stimulation buffer containing various concentrations of ligand were added. After incubation at 37°C for 1hr, 5 μ L IP1-d2 conjugate followed by 5 μ L of Eu-cryptate labeled anti-IP1 antibody were added. Time-resolved fluorescence at 620 nm and 665 nm were measured with PHERAstar FS after incubation at 4°C overnight, and the ratios of the signals and Delta F were calculated.

Delta F % = (Standard or sample Ratio - Ratio_{neg})/Ratio_{neg} x 100

Results & Discussion

As shown in table 1, IP-One has already been validated on different models and targets - even if the cell lines involved were not specifically optimized for IP-One assay.

Table 1: List of GPCRs already validated with IP-One assay for agonist responses. The GPCR expression in the cell is stable (s), transient (t) or endogenous (e). HTRF® IPOne assays were performed on BMG LABTECH's plate reader.

GPCR Target	Cell Line	Agonist	EC ₅₀ HTRF® IP-One	EC ₅₀ Isotopic method
Muscarinic M1/Gq (s)	CHO-K1	Acetylcholine	71 nM	42 nM
		Carbachol	296 nM	300 nM
Vasopressin V1A/Gq (s)	CHO-K1	Vasopressin	1 nM	0.4 nM
		Vasopressin	1.6 nM	0.4 nM
Oxytocin OT/Gq (s)	CHO-K1	Oxytocin	13 nM	7 nM
Histamin H2/G16 (s)	CHO-K1	Amthamine	21 nM	16 nM
Purinergic P2Y1/Gq (s)	1321N1	2-methylthio ADP	6.8 nM	n.d.
Cholecystokinin CCK1/Gq (s)	1321N1	CCK8 sulfated	2 nM	43 nM
Chemokine CCR5/G16 (s)	CHO-K1	RANTES	76 nM	26 nM
		MIP1 alpha	48 nM	n.d.
HupCar/Gq (s)	CCL39	Calcium	2.9 mM	n.d.
Endothelin Etb/Gq (s)	CHO-K1	Endothelin 2	82 nM	83 nM
		Ala-Endothelin	70 nM	93 nM
TRH1/Gq (s)	CHO-K1	TRH	0.8 nM	n.d.
GB1+GB2/Gqj9 (t)	HEK293	GABA	980 nM	484 nM
mGluR 1/Gq (t)	HEK293	Quisqualate	113 nM	75 nM
mGluR 5/Gq (t)	HEK293	Quisqualate	13 nM	9 nM
Muscarinic M3/Gq (e)	HEK293	Acetylcholine	20 μ M	n.d.
		UTP	2.1 μ M	n.d.
Purinergic P2Y1/Gq (e)	HEK293	UTP	2.1 μ M	n.d.
		ATP	1.6 μ M	n.d.

The validations also conclusively demonstrate the assay's performances in the presence of cell lines using Ga16 or Gqj9 type chimeric constructions.

The IP-One assay showed equally good performance on different cellular backgrounds, stable or transient transfected cells, and chimeric constructs. There is a strong correlation with reference methods and no cross reactivity with 50 μ M of the following [phospho] inositides phosphates could be observed: Myo-inositol, PIP2, PIP3, IP2, IP3 and IP4.

In addition, the IP-One assay was applied upon the 1321N1-CCK1 cell line together with the agonist CCK8-sulfated. The experiment was performed in 96-well plates resulting in very close EC₅₀ values and a very good correlation regarding the %inhibition/basal curve (figure 2). Z' calculations at an agonist concentration equal to EC₅₀ resulted in Z' > 0.78.

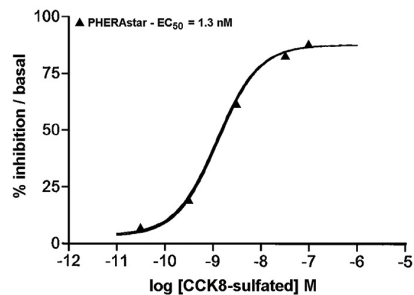


Fig. 2: HTRF® IP-One assay. The 1321N1-CCK1 cell line and CCK8-sulfated as agonist were applied.

Calcium measurement is often not sensitive enough to specifically detect the inhibition of the GPCR constitutive activities, whereas a modulation in the concentration of IP1 is perfectly able to show this.

The messenger's stability opens the possibility of the IP-One assay's application to cases where other HTS technologies fail to provide a satisfactory solution - such as in the detection of inverse agonist activities. The assay also profits from the special qualities of d2, one of the latest improvements implemented in HTRF® technology.

Conclusion

Cisbio's new IP-One assay, which is based on its proprietary HTRF® technology, is the first high throughput system that can easily detect inositol(1)phosphate (IP1), which tightly correlates with Gq-coupled activity.

All HTRF® IP-One assay results were produced on BMG LABTECH's the multimode plate reader PHERAstar FS. The reader showed strong results in terms of EC₅₀ and Z' values.



PHERAstar® FSX

*The PHERAstar FSX is the newest PHERAstar reader.



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