HTRF® IP-One Assay Performed on the PHERAstar and RUBYstar Plate Readers

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- HTRF® IP-One assay for Gq pathway investigation under HTS conditions
- Comparable performance of PHERAstar and RUBYstar with the HTRF® IP-One assay 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 Gαs or Gαi 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) tri-phosphate (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. This of course has tended to make its detection highly inaccurate. 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 dedicated HTRF® plate reader RUBYstar and the multimode plate reader PHERAstar from BMG LABTECH. These HTRF® certified compatible readers are 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. Such technological efficiency, combined with the use of a high-affinity monoclonal antibody, give the assay all the qualities necessary for an HTS tool, particularly in terms of sensitivity, robustness and reliability.

Assay Principle

As previously mentioned, IP3, though, being the top choice as a secondary messenger for the Gq signaling pathway, nevertheless does not possess the necessary profile for an HTS tool because of its overly-brief physiological existence. Moreover, there is no inhibitor available for the phosphatases which convert IP3 into IP2 and then into IP1. On the other hand, 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.

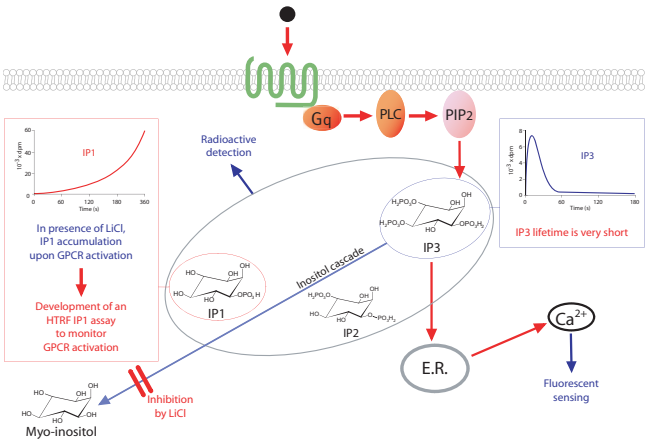

Fig. 1: Ways to monitor Gq coupled GPCR activation: Gq coupled receptor activation induces IP3 release catalysed by PLC. IP3 degradation occurs rapidly and leads ultimately to the production of IP1. Processing of IP1 into myo-inositol can be prevented by the addition of LiCl.

Materials and 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 (figure 2). 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 then start to 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 µL total assay volume. The HTRF® signal was read on the PHERAstar and the RUBYstar.

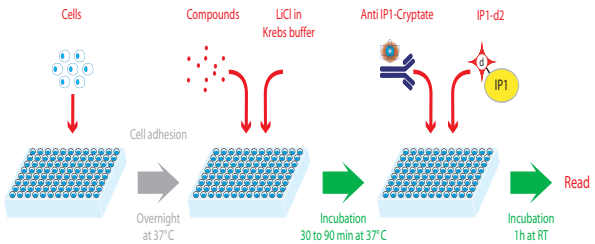

Fig. 2: The IP-One assay protocol comprises two incubation steps – cell stimulation and IP1 detection – and can be run in a single culture microplate. The quantification process involves only the addition of the two conjugates and takes no longer than one hour.

Cells were kindly provided by the Institut de Genomique Fonctionnelle (IGF), Montpellier, France and Euroscreen, Gosselie, Belgium. IP-One kit was supplied from Cisbio international (France). The dedicated plate reader RUBYstar and the multimode plate reader PHERAstar were purchased from BMG LABTECH (Germany).
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 µL/well. After incubation at 37°C, the culture supernatants were completely discarded. Then immediately after, 10 µL of stimulation buffer containing various concentrations of ligand were added. After incubation at 37°C for 1hr, 5 µL IP1-d2 conjugate followed by 5 µL of Eu-cryptate labeled anti-IP1 antibody were added. Time-resolved fluorescence at 620 nm and 665 nm were measured with PHERAstar and RUBYstar 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 and 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). EC_{50} were determined experimentally and in some of the cases in a side by side comparison with IP isotopic detection. HTRF® IP-One assays were performed on BMG LABTECH's dedicated HTRF® plate reader RUBYstar.

GCPR 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
Cholecystokinine 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 / Gqi9 (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 µM	n.d.
Purynergic P2Y1 / Gq (e)	HEK293	UTP	2.1 µM	n.d.
		ATP	1.6 µM	n.d.

The validations also conclusively demonstrate the assay's performances in the presence of cell lines using Gα16 or Gqi9 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 µM of the following (phospho) inositides phosphates could be observed: Myo-inositol, PIP2, PIP3, IP2, IP3 and IP4.

In addition, for a direct performance comparison of BMG LABTECH's PHERAstar with the RUBYstar 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 EC_{50} values and a very good correlation regarding the %inhibition / basal curve (figure 3). Z' calculations at an agonist concentration equal to EC_{80} resulted in Z' › 0.78 for both plate readers.

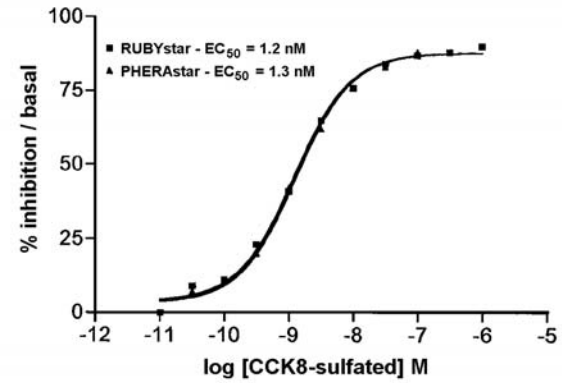

Fig. 3: Direct performance comparison of the PHERAstar with the RUBYstar using the HTRF® IP-One assay. The 1321N1-CCK1 cell line and CCK8-sulfated as agonist were applied.

But reaching beyond these expected results, the IP-One assay has already proved its worth in the field of screening for inverse agonist compounds. Looked at closely, and given its transitory characteristics, 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. Using the IP-One assay therefore represents a tempting new alternative when investigating Gq-coupled receptors via the quantification of a new secondary messenger for this pathway, IP1. 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. Given its performance, d2 is now included in all HTRF® cAMP assays. The new HTRF® platform for GPCR screening will thus incorporate a series of HTS assays, all using similar protocols and a standard robotics and detectors. Such a platform obviously holds all that is needed to provide test solutions which are particularly well-adapted to meeting the challenges of the different stages of drug screening.

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), one of the major products of the phosphatidyl inositol cascade, which tightly correlates with Gq-coupled activity. In association with Cisbio's cAMP assay, it allows the establishment of a complete HTRF® based GPCRs platform fully covering the different signalling pathways triggerred by GPCRs. All HTRF® IP-One assay results were produced on BMG LABTECH's dedicated HTRF® plate reader RUBYstar and the multimode plate reader PHERAstar. The readers showed in a comparison strongly correlating results in terms of EC_{50} and Z' values.

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