HTS instrument discovers low affinity inhibitors of the Inositol Phosphate (IP) signaling pathway

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- **Several low affinity drug 'hits' were only found by the PMT-based reader and not the CCD-based reader**
- **PHERAstar® *FS* performs more than twice as fast as the CCD-based reader**
- **PHERAstar *FS* shows superior assay quality parameters such as Z', DeltaF% and assay window**

Introduction

G-protein coupled receptors (GPCRs) are transmembrane proteins which play a key role in the signal transduction of extracellular stimuli. GPCRs are associated with a complex assembly of intra-cellular proteins regulating a large variety of downstream effectors. The production of inositol 1,4,5-triphosphate (IP3) is one such second messenger, which is produced in response to the activation of Gq-coupled receptors. However, IP3's very short half-life make its assessment too challenging for drug screening assays and the monitoring of calcium release, triggered by IP3, has been extensively used as a downstream readout of this signaling pathway.
An alternate way to monitor IP3 is to measure the accumulation of inositol monophosphate (IP1), which is a downstream metabolite of IP3.

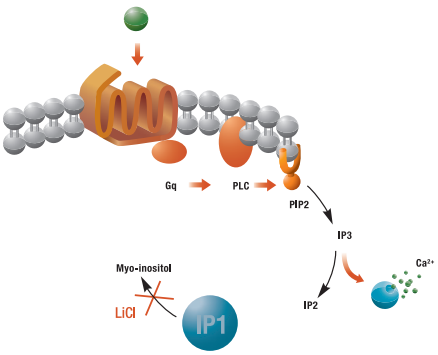

Fig. 1: Biosynthesis of IP1.

Monoclonal antibodies raised against IP1 lead to the optimization of a homogeneous time-resolved fluorescence assay (HTRF®), taking advantage on the fact that IP1 is stable and accumulates in cells. The HTRF® IP-One assay has been compared to existing methods and has been shown to lead to similar compound potency data. Moreover, the end point accumulation of IP1 allows for the discrimination of slow acting compounds that remain unseen by calcium sensing. The HTRF® IP-One assay also allows for the characterization of inverse agonists by the quantification of constitutively active GPCRs, which is impossible via measurement of calcium release. Lastly, HTRF® IP-One detection confers superior assay robustness and much lower false positive rates compared to calcium detection. The IP-One assay developed by Cisbio Bioassays uses their proprietary HTRF® technology (Figure 2).

Assay Principle

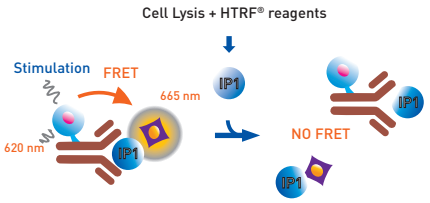

Fig. 2: IP-One HTRF® Assay Principle.

The assay uses a monoclonal antibody that specifically recognizes IP1 and it is based on a competition format in which the intracellular accumulation of IP1 inhibits the fluorescence resonance energy transfer (FRET) signal between the HTRF® donor and acceptor. An IP1 calibration curve can estimate the IP1 concentration accumulated in cells as a function of the compound concentration.

Materials & Methods

- Next generation PMT based HTS microplate reader, PHERAstar *FS*
- HTS CCD-based microplate imager from a different vendor

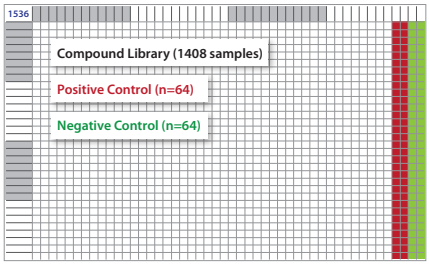

Fig. 3: 1536-well microplate layout.

Black Greiner 1536-well microplates were used with an assay volume of 5 µL. As shown in Figure 3, 1408 different compounds were pipetted into the first 44 columns of a microplate, with positive (POS, n=64) and negative (NEG, n=64) controls in the last 4 columns.
The PHERAstar *FS* is equipped with a high power pulsed nitrogen laser emitting at 337 nm, as well as a dedicated Simultaneous Dual Emission (SDE) direct photon counting time-resolved fluorescence mode. When exciting the terbium (Tb) donor molecule, the

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laser is superior to a broadband xenon flash lamp. The laser's energy emission takes advantage of the higher molecular extinction coefficient of the terbium cryptate peaking around 337 nm, compared to europium (Eu).

Results & Discussion

Results were evaluated by the HTRF® ratio of the two emission wavelengths (Em 665 nm / Em 620 nm) and hits are shown as peaks in a surface graph representing the entire 1536 well microplate.

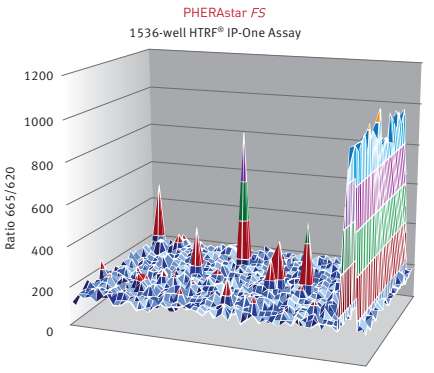

Fig. 4: HTRF® ratios obtained for the IP-One assay with the PHERAstar *FS*.

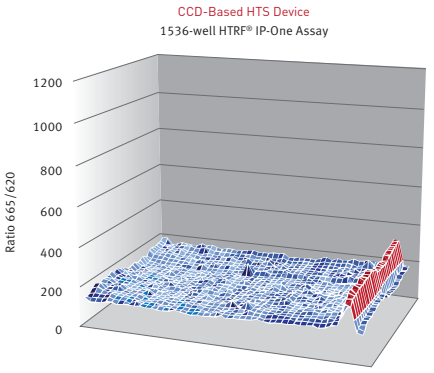

Fig. 5: HTRF® ratios obtained for the IP-One assay with a CCD-based HTS Device.

Table 1: Speed and assay quality are compared between the PHERAstar *FS* and the CCD camera based HTS reader.

	PHERAstar *FS*	CCD based HTS reader
Read Times (1536)	53 sec	2:12 min
Assay Window	6:1	2:1
Delta F%	490	76
Z' Value	0.70	0.24

Conclusion

The IP-One HTRF® assay from Cisbio was performed on two different HTS microplate readers with different detection technologies. Low affinity compounds which were not discovered with a leading HTS CCD camera based imaging microplate reader were readily resolved with the PMT based PHERAstar *FS*. This next-generation HTS reader, the PHERAstar *FS* from BMG LABTECH, represents a new choice for HTS screening assays.

PHERAstar® *FSX*

*The PHERAstar *FSX* is the newest PHERAstar reader.