

HTS Instrument Discovers Low Affinity Inhibitors of the Inositol Phosphate (IP) Signaling Pathway

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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 intracellular 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.

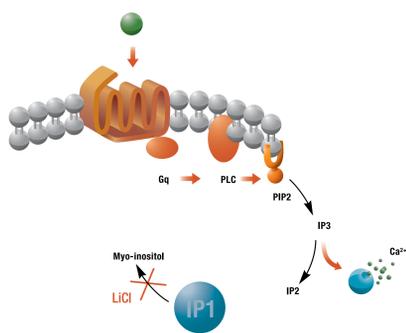


Fig. 1: Biosynthesis of IP₁.

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 comparable 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

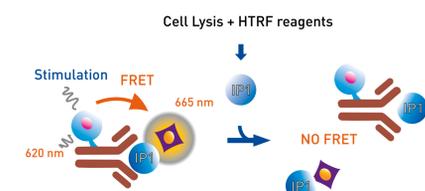


Fig. 2: IP-One HTRF[®] Assay Principle

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.

Results and 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.

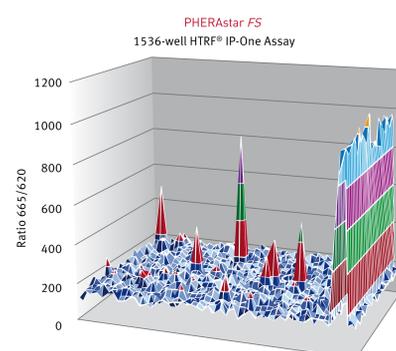


Fig. 4: HTRF[®] ratios obtained for the IP-One assay with the PHERAstar FS

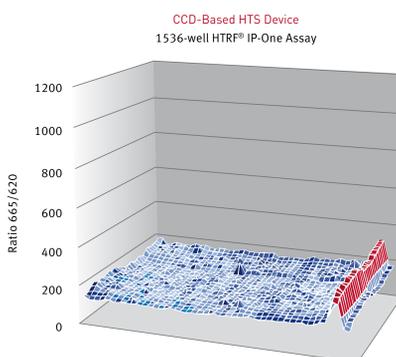


Fig. 5: HTRF[®] ratios obtained for the IP-One assay with a CCD-based HTS device

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

This next-generation HTS microplate reader, the PHERAstar FS from BMG LABTECH, represents a superior choice for HTS screening assays compared to CCD-based imagers.



Fig. 6: BMG LABTECH's high-end multidetection microplate reader PHERAstar FS

Materials and Methods

- Next generation PMT based HTS microplate reader, PHERAstar FS (BMG LABTECH)
- HTS CCD-based microplate imager from a different vendor

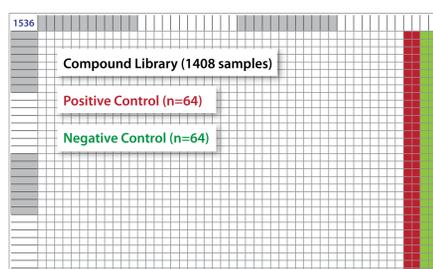


Fig. 3: 1536-well microplate layout

Black Greiner 1536-well microplates were used with an assay volume of 5 μ L. 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 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).

Summary

Follow up analysis has shown that classic high-throughput screening (HTS) platforms have not yielded the number of marketable drugs as once thought. Furthermore, most drugs that do go to market have low molecular weights (<500 Da). This suggests that classic HTS screens, which are performed at lower volumes and concentrations, seem to be missing lower affinity drug targets. As a consequence different technologies, such as NMRs, are being used to detect lower affinity compounds or compound fragments. However, there is still considerable interest in using classic HTS bioassays to look for low affinity compounds.

Using the IP-One HTRF[®] assay from Cisbio, which measures the lower response second messenger inositol monophosphate (IP₁),

a downstream metabolite of inositol 1,4,5-triphosphate (IP₃), two different HTS microplate readers with different detection technologies were assessed.

The PHERAstar FS, a next-generation photomultiplier (PMT) based instrument was compared to an industry leading charged coupled device (CCD) camera based imaging instrument.

The data herein shows the PMT reader when compared to the CCD reader:

- Found several lower affinity drug 'hits'
- Had superior Z', assay window and Delta F%
- Measured >2x as fast