HitHunter® IP3 assay for GPCR screening using the PHERAstar® *FS*

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- **PHERAstar® *FS* multimode high-throughput microplate reader was used to access Gq coupled GPCRs**
- **HitHunter® is a robust and sensitive homogeneous fluorescence polarization assay to measure IP3 levels**
- **Assay miniaturization up to 1536-well plate format**

Introduction

G protein coupled receptor (GPCR) activation regulates cell signaling via several second messengers, including 3'-5'-cyclic AMP (cAMP), inositol phospholipids and calcium. The quantitation of accumulation of these second messengers is used to pharmacologically characterize both the action of GPCR ligands and to identify novel compounds in high-throughput screening (HTS). GPCRs coupling to $G\alpha_s$ and $G\alpha_i$ proteins activate or inhibit, respectively, adenylate cyclase, subsequently changing intracellular cAMP levels. GPCRs coupling to $G\alpha_q$ or $G\alpha_o$ proteins activates phosphoinositol phospholipase C-ß, an enzyme that hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to form *sn*-1,2-diacylglycerol and inositol- 1,4,5-trisphosphate (IP3). IP3 binds and opens endoplasmic IP3 gated calcium channel, resulting in the release of bound calcium into the cytosol. Metabolic products of IP3 also modulate cell signaling, such as inositol 1,3,4,5-tetrakis-phosphate (Ins P4), which synergistically facilitates IP3, mediated calcium release. DiscoverX has developed a homogeneous assay based on fluorescence polarization (FP), to measure IP3 levels generated by GPCR activation. This application note, describes the use of IP3 assay along with PHERAstar *FS* multimode HTS plate reader.

The assay is based on FP mode using a proprietary binding protein that is highly selective for the active isomer of IP3. The assay is a competitive binding assay (figure 1), in which cellular IP3 displaces a fluorescent derivative of IP3 from a specific binding protein. The assay measures changes in FP, a single wavelength ratiometric technique, in which a fluorescent derivative of IP3 is used as a tracer. In the assay unlabelled IP3, either a standard IP3 solution, or derived from the cell lysate, displaces the tracer from the binding protein and the rotation time increases and low FP signal is measured. By this means, a calibration is generated to the standard IP3 dilutions and the molar concentration of IP3 in the cell lysate is determined by interpolation.

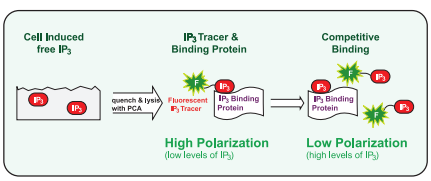

Fig. 1: HitHunter® IP3 fluorescence polarization assay principle.

Materials & Methods

BMG LABTECH's PHERAstar *FS* combines rapid plate reading necessary for HTS with the enhanced performance and sensitivity needed to read small fluid volumes. The multimode microplate reader has the flexibility to excel with the most demanding assays and is designed to read all leading HTS detection modes (fluorescence intensity, time-resolved fluorescence, fluorescence polarization, luminescence and absorption) in all formats up to 1536. The PHERAstar *FS* was run in FP mode for the monitoring of the HitHunter® IP3 demo kit (DiscoverX Corp.) containing the following reagents:

HitHunter IP3 kit reagents	
1	IP3 Standard – 20 µM
2	IP3 Standard Dilution Buffer
3	Perchloric Acid (PCA) – 0.2 N
4	IP3 Tracer - Green
5	IP3 Binding Protein

The standard curve for DiscoverX's IP3 kit was run according to the package insert protocol in black 384-well plates (non-binding polypropylene plates; Greiner), and the fluorescence polarization signal was read on the PHERAstar *FS* one hour after the addition of the last reagent using the following protocol for plate reader setup (figure 2).

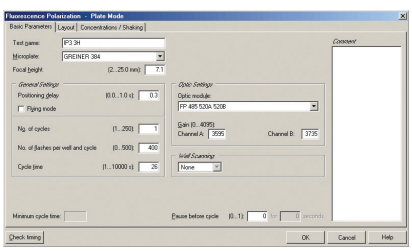

Fig. 2: This screenshot shows the HitHunter® IP3 FP assay setup window from the PHERAstar *FS*. An optical module for HitHunter® IP3 is directly available from BMG LABTECH.

The table below outlines the volumes and procedures for the IP3 protocol measuring both standard curve and agonist/antagonist treated cells in a 384-well plate format. Measuring agonist/antagonist treated cells, IP3 levels were stabilized with the addition of 5 µL of 0.2 N perchloric acid.

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Table 1: HitHunter® IP3 protocol for standard curve and agonist/ antagonist treated cells.

Hit Hunter IP3 Protocol			
Condition	**Standard Curve**	**Cells Agonist**	**Cells Antagonist**
Step 1: Standard/ cells	10 µL standard	10 µL cells	10 µL cells
Step 2: Antagonist	-	-	5 µL antagonist
Incubate	-		30 min. at 37° C
Step 3: Agonist	5 µL water	5 µL agonist	5 µL agonist
Incubate	-	20 seconds (room temperature)	
Step 4: PCA	5 µL PCA -0.2N		
Step 5: Tracer	10 µL IP3 Tracer-Green		
Step 6: Binding Protein	20 µL IP3 Binding Protein		
Gently tap plates for even mixing (shake plates for 5 minutes)			
Read fluorescence polarization signal on the PHERAstar *FS* (Excitation filter 485 nm/Emission filter 520 nm)			

Results & Discussion

DiscoverX Hithunter® IP3 was prepared in 384-well format and standard curves were run on BMG LABTECH's PHERAstar *FS* in fluorescence polarization mode. IP3 standard reagents were added according to the assay protocol and the fluorescence polarization signal was read one hour after the addition of the last reagent. The Hithunter® IP3 standard curve is illustrated in the figure below (figure 3).

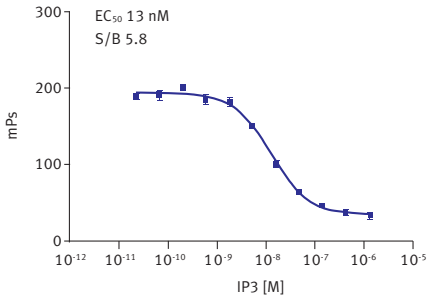

Fig. 3: Hithunter® IP3 Assay: Standard curve data in 384-well format.

The Hithunter® IP3 assay is a robust, sensitive and specific tool for measuring cellular D-myo-inositol 1,4,5-triphosphate. The signal is based on competitive binding between an IP3 fluorescent tracer and an unlabeled IP3 from cell lysate or IP3 standard. The signal is read as a change in fluorescence polarization and is inversely proportional to the amount of IP3 in cell lysates. The standard curve signal to background is >5, and the EC_{50} is 13 nM. The signal can be measured immediately or up to 16 hours later.

Conclusion

GPCRs are critical targets in HTS drug discovery and GPCR signaling can be examined by direct quantitation of IP3 by applying the HitHunter® IP3 kit. Good results were obtained on the PHERAstar *FS* multimode microplate reader which is designed to read all leading HTS detection modes in formats up to 1536. The high degree of sensitivity, easy-to-use software, robust hardware and optimized detection systems make the PHERAstar *FS* ideal for GPCR analyses in the high-throughput assay environment.

The PHERAstar was run in FP mode for the HitHunter® IP3 assay, which uses FP for sensitivite detection and ease of use. The assay is ideal for the detection of basal levels of IP3 in cell lysates, as well as low levels for IP3 induction. Once the cells are lysed, the stable assay signal can be read the same day or overnight. There are few robust IP3 assays currently available and none easily scalable to 1536 automation. DiscoverX offers this assay to give maximum precision and reliability for GPCR screening.

For more information on DiscoverX assays please refer to the web site: www.discoverx.com

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

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