HTRF® IP-One assay used for functional screenings

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- **Single cell clones functional screening using fresh and frozen cells**
- **Functional HTS for the MRCT 100K compound collection**
- **Detection for screening is simplified using the HTRF optic module**

Introduction

G protein coupled receptors (GPCR) are a most prominent group of therapeutic targets. In most cases, the activation of GPCRs results either in the alteration of cellular cAMP level or in the release of calcium ions from intracellular stores. Various assays are available to determine receptor activation by direct or indirect quantification of these second messengers.
Cisbio Bioassays developed highly accurate HTRF® assays for measuring second messengers like IP1 or cAMP in HTS formats. This application note demonstrates the use of the IP-One HTRF® assay for a functional selection of clones from stably transfected recombinant CHO-M1 cells. Next to this, results from a high-throughput screen of Galanin receptor GALR2 are presented. This screen employed the MRCT 100K compound collection, a selection of drug-like molecules from commercial libraries which includes over 10,000 compounds which target protein-protein interaction.

Assay Principle

A robust HTRF® functional IP1 assay (Cisbio) was used for screening. The assay is based on a competitive immunoassay principle whereby free IP1 competes against IP1-d2 (HTRF® acceptor) for binding to anti-IP1 Cryptate conjugate (HTRF® donor). The signal is inversely proportional to IP1 levels in the cell with maximum FRET obtained in the absence of IP1 (Figure 1).

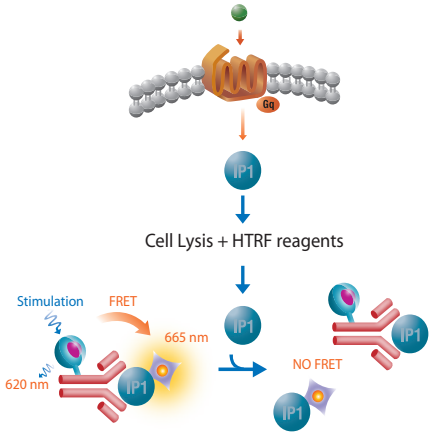

Fig. 1: Cisbio IP-One HTRF assay.

Materials & Methods

- CHO cells stably expressing GalR2 (GE Healthcare)
- Fresh and Frozen CHO-M1 Cell lines, CCS Culture Service, Germany
- IP-One HTRF® assay kit (Cisbio)
- White 384-well small volume plates (Greiner)

Single cell clone screening
CHO-K1 cells were transfected with an expression vector encoding the Gaq-coupled human muscarinic acetyl-choline receptor M1 (CHRM1). Stably transfected monoclonal cell lines were isolated by selection with G418 in 384-well plates. In order to screen the clones for a functional agonist response, 10,000 cells were seeded into white 384-well plates and were left to adhere overnight. The next day the medium was discarded and cells from each clone were stimulated with agonist for 1 hour while identical cells in a control well were left untreated. Cells were prepared according to a standard freezing protocol and used in the assay as follows: After thawing, the cells were washed once in culture medium and resuspended in IP1 stimulation buffer. To compensate for the preincubation time of the fresh cells, which grew over night, the frozen cells were seeded at double density of 20,000 cells per well. The agonist was added to the cells in suspension immediately after seeding.

GalR2 screening
The assay was configured using CHO cells stably expressing GalR2. Pre-incubation of cells with a sub-maximal concentration of the galanin agonist sensitised the HTS to the simultaneous detection of both agonists and PAMs. The MRCT 100K compound collection was screened at a final assay concentration of 10 µM. 5 µl of cells/well (15,000 cells) were dispensed and 2.5 µl compound or buffer control was added to 384 well low volume white plates. Following a 30 min incubation at 37°C, 2.5 ml of galanin was added at a maximal concentration of 1 µM (EC100) or an above-minimal concentration of 3.16 nM (EC20). Test samples received buffer containing 0.1% BSA. Plates were incubated for 1 hr at 37°C.

HTRF® IP-One assay on PHERAstar® *FS*
The IP-One HTRF® assay was performed as recommended by Cisbio. Briefly, donor and acceptor were added consecutively to the cells followed by 1 hour incubation at room temperature. Plates were then put in the PHERAstar *FS* microplate reader. The 665 and 620 nm signals were measured simultaneously by using the HTRF specific optic module.

Rev. 12/2014
Keywords: **GPCR, HTRF, IP-One, Receptor, Screening**

Data calculation

Data normalization was performed by calculating the ratio of the raw data obtained at 620 and 665 nm:
Ratio = (665/620)*10000

Results & Discussion

Single cell clone screening

Upon stimulation with the physiological ligand acetylcholine (1 µM), recombinant CHO-M1 clones were functionally screened by the IP-One HTRF® assay. (Figure 2).

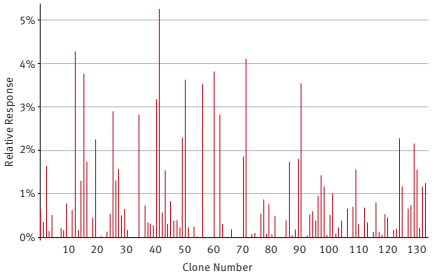

Fig. 2: Screening of single cell clones. Data are displayed in relation to the IPOne standard curve (29 µM IPOne = 100 %).

Out of 134 clones 27 were exhibiting a significant response to the agonist with different sensitivity. One selected cell line (clone B2) was taken to do a dose response curve with carbachol, a partial agonist (Figure 3).

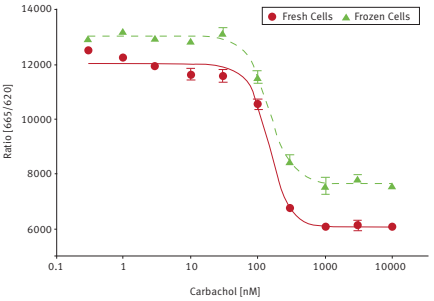

Fig. 3: Dose response of carbachol with cells from a growing culture (fresh cells) and from frozen cells.

Although the signal to background ratio was slightly reduced in frozen cells, similar EC50 values were determined (147 nM for fresh cells and 149 nM for frozen cells). The Z'factor of the assay with frozen cells was still very good (> 0.7).

GalR2 screening

The 665/620 ratio was expressed relative to EC100 and EC20 such that EC20 = 0% and EC100 = 100% (Figure 4). Hits were selected using a 30% response cut off or 30% above EC20.

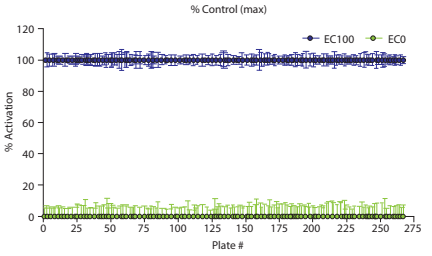

Fig. 4: Data were normalized to high (EC100) and low (EC20) controls.

The HTS assay performed well as indicated by the robust and consistent Z' data. For 85320 compounds screened, a mean Z' of 0.72 (± 0.05) was obtained. The number of hits is presented in Table 1.

Cutoff (%)	# Hits
30	250
40	117
50	65
60	29
70	13
80	9
90	4
100	1

The screen gave a fairly low hit rate (0.3 % at 30 % activity).

Conclusion

The IP-One HTRF® assay from Cisbio proved to be a fast and reliable method to directly screen a large number of samples using the PHERAstar *FS* microplate reader. A robust assay could be established using fresh cells from a growing culture as well as Frozen Instant Cells.