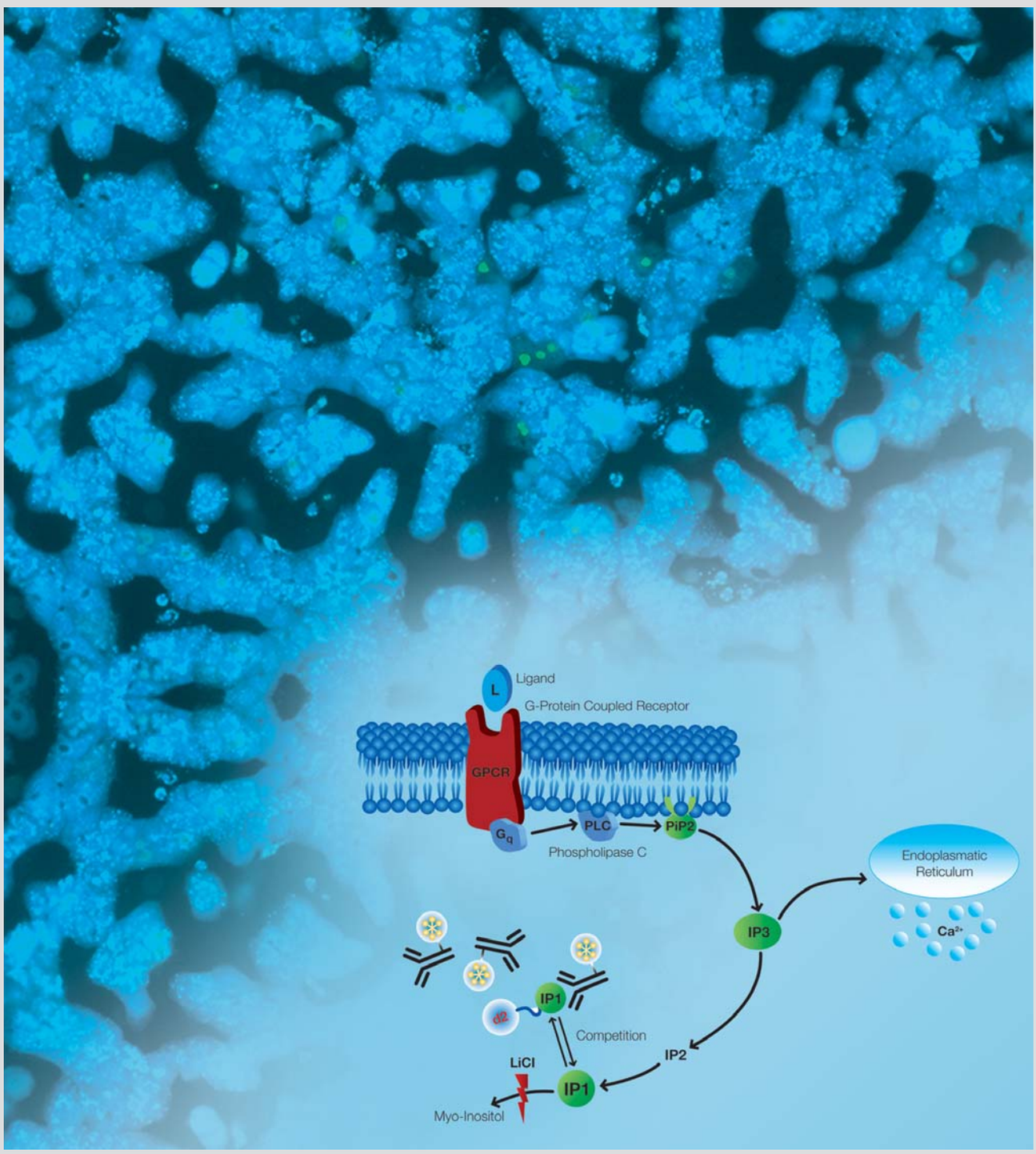
Application Note

Establishing a cell culture assay based on time-resolved fluorescence resonance energy transfer (TR-FRET) for screening G-Protein coupled receptors



greiner bio-one

Introduction

Time-resolved fluorescence resonance energy transfer (TR-FRET) has become a popular technique in the field of high-throughput screening. Its popularity is mainly due to the high sensitivity[1], the lack of any radioactive reagents and the health and safety issues these cause. TR-FRET is based on the transfer of photons between a lanthanide complex, the donor to a suitable acceptor, when they are in close proximity. The lanthanide donor complex exhibits a long fluorescence lifetime with a shallow signal decay curve. When this donor complex is excited by a pulsed light source, e.g. flash lamp or fluorometer laser, its extremely long lifetime allows the separation of this signal from the light emitted by other fluorophores with a normal, shorter lifetime (**Fig. 1**). Together with the large Stoke's shift of the lanthanides fluorescence and the ratiometric nature of the readout, interference from false-positive arising from autofluorescent compounds in the screening collection is drastically reduced[2].

Amongst the different commercial TR-FRET kits available Cisbio's technology is one of the most popular[3]. The HTRF (homogeneous time-resolved fluorescence) technology is dedicated to high-throughput drug screening and lead generation and has been extensively used as a tool for screening a number of tyrosine kinases alongside measuring other molecular complexes.

The latest development from Cisbio is the introduction of the IP-One HTRF assay, a cell-based functional assay for the monitoring of phospholipase C coupled (PLC) receptors and the validation of the function of various G-protein coupled receptors (GPCRs)[4]. G-protein coupled receptors (GPCRs) are a large protein family of transmembrane receptors that react with specific extracellular molecules, activate inside signal transduction pathways and, ultimately, cellular responses.

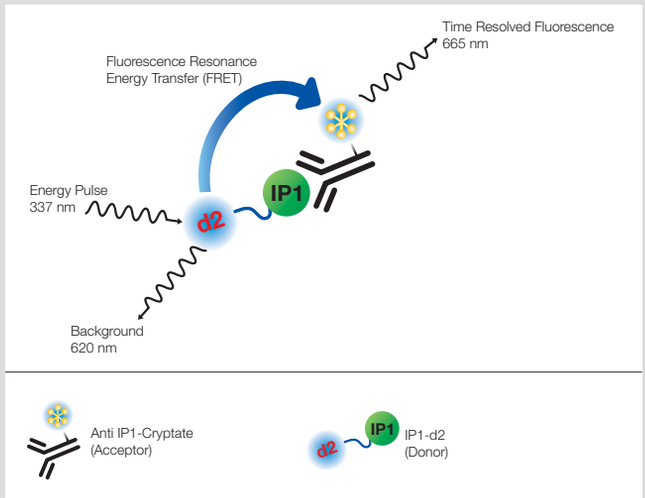


Figure 1: Mechanism of HTRF
Homogeneous Time-Resolved Fluorescence uses two fluorophores, a donor and an acceptor. Excitation of the donor by an energy source (e.g. flash lamp or fluorometer laser) triggers an energy transfer to the acceptor if they are within a given proximity to each other. The acceptor in turn emits light at its given wavelength.

G-protein coupled receptors are involved in many diseases and are the target of about 25 % of the world's top selling drugs. They also represent approximately one third of the number of targets currently being investigated by the pharmaceutical industry[5].

GPCRs can be classified into 3 subclasses based on the secondary messenger they affect. Gαs or Gαi coupled GPCRs regulate cAMP levels, while Gq coupled GPCRs activate phospholipase C (PLC) and trigger the inositol phosphate (IP) cascade (**Fig. 2**).

Several metabolites in this pathway, including IP3, have extremely short half lives, making them difficult to accurately quantify. IP-One, a downstream metabolite of IP3, accumulates in cells following Gq receptor activation and is stable in the presence of LiCl inhibiting the conversion of IP1 into myoinositol by inositol monophosphatase[4].

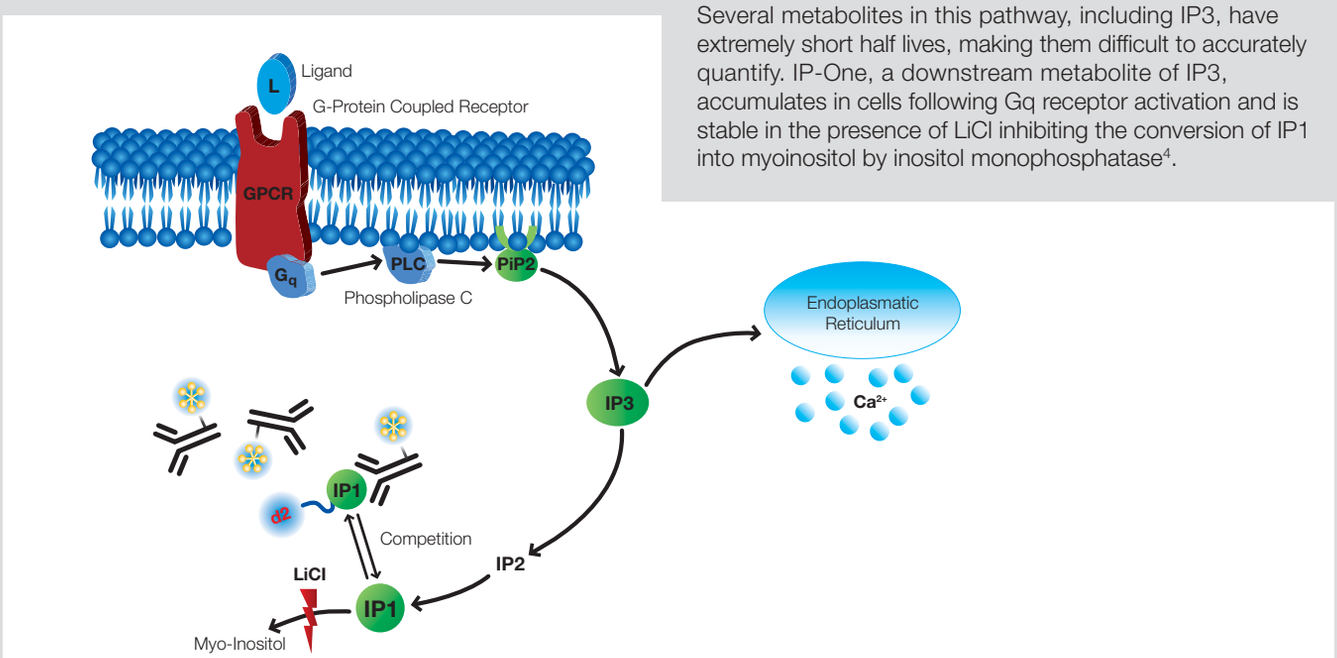


Figure 2: IP-One HTRF assay principle

Material and Methods

Assay technology

G-protein coupled receptor screening was established based on the IP-One HTRF technology (Cisbio, France). The IP-One HTRF assay is based on a monoclonal antibody, specific to IP1, labelled with europium cryptate which competes with both native IP1 produced by cells and IP1 coupled with the HTRF acceptor (d2). The specific signal (energy transfer) is inversely proportional to the concentration of IP1 in the calibrator or cell lysate (**Fig. 2**).

The experiment was conducted following the recommended assay protocol (**Fig. 3**).

Tissue culture

Cells were cultured according to standard procedures in 175 cm^2 tissue culture flasks (Cat.-No. 660 175, Greiner Bio-One GmbH, Frickenhausen, Germany) in Earle's MEM medium (Biochrom AG, Berlin, Germany) containing 10 % fetal horse serum, 2 % glutamine, 10 ml non essential amino acids (50 x, Biochrom AG, Berlin, Germany), pyruvate (Biochrom AG, Berlin, Germany) in a humidified atmosphere at 5 % CO_2 and 37°C.

Cultures were split (1:10) every 4 days using standard trypsination procedures (0.05 % trypsin containing 0.02 % EDTA solutions from Biochrom AG, Berlin, Germany). Medium was changed after 2 days.

For the preparation of the assay plates cells were grown to 80 % confluence and concentrated in cell culture media in a 50 ml polypropylene tube (Cat.-No. 227 261, Greiner Bio-One GmbH, Frickenhausen, Germany) to a final concentration of 1,000 cells / µl.

Preparation of assay plates

The validation of the IP-One cell based assay was carried out in white cell culture treated microplates from four different manufacturers. Before using these microplates background was determined under standard HTRF conditions. The excitation wavelength used was 337 nm with emission wavelengths read at 665 and 620 nm in a BMG time-resolved fluorescence reader (Pherastar, BMG LabTech, Offenburg, Germany). The measurement at 620 nm represents the background of the assay whereas the results at 665 nm represent the time-resolved fluorescence of the acceptor.

Each well that was used in the assay was seeded at 40,000 cells / well (assay volume of 40 µl) and the plates were incubated overnight at 37°C allowing the cells to attach to the cell culture treated surfaces.

The following steps, including all controls, were conducted according to the protocol supplied by Cisbio (**Fig. 3**).

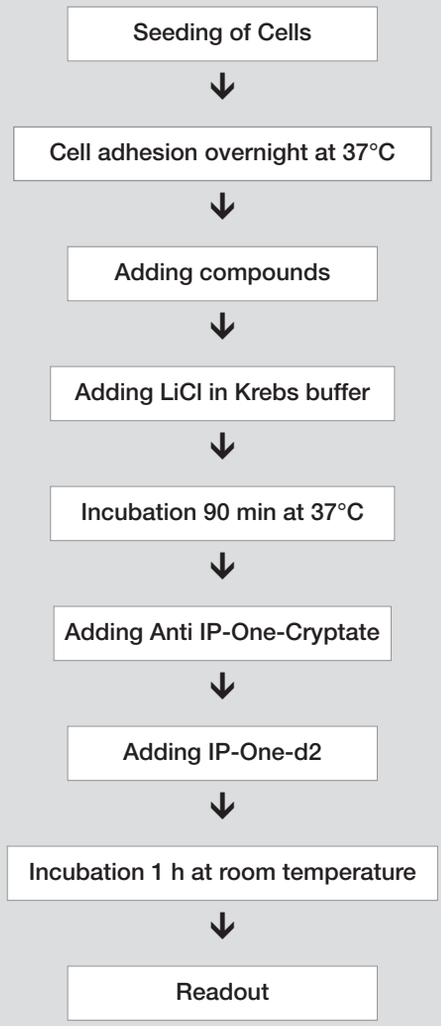


Figure 3: Overview of the assay procedure

Stimulation

Cells were stimulated with a ligand which acts as an acetylcholine-receptor agonist and stimulates both muscarinic and nicotinic receptors.
For establishing dose – inhibition curves different ligand concentrations (1.14 / 3.43 / 10.29 / 30.86 / 92.59 / 277.78 / 833.33 / 2500 µM ligand) were used.

Analysis

Microplates were read one hour after adding the HTRF reagents in a Pherastar microplate reader (BMG LabTech, Offenburg, Germany) with standard HTRF conditions. The ratios of the readouts were calculated according to the formula

Ratio = (Emission at 665 nm / Emission at 620 nm) x 10,000

Results and Discussion

Influence of the background of microplates on the assay result

To determine if background fluorescence may have any influence on the assay performance, empty microplates were measured under usual TR-FRET conditions before cell seeding (**Table 1**).

One of the microplates under examination (competitor 3) had a background approximately three times higher than all the other microplates on test. The elevated background signal was detected at both emission wavelengths (620 nm and 665 nm). Microplates from Greiner Bio-One showed low background signals and coefficient of variation, whereas competitor 1 and 2 had CVs above 10 %.

Assay controls as indicator for assay stability

The IP-One test kit includes several internal controls[4]. One of these controls consists of standard solutions with defined concentrations of non labelled IP1. These standards were used to calibrate the homogeneity of our pipetting process in a dose dependent time-resolved signal. The standards showed perfect alignment and a nice dose response curve for all four tested plates indicating that the assay worked well and that the pipetting procedure was homogeneous (**Fig. 4**).

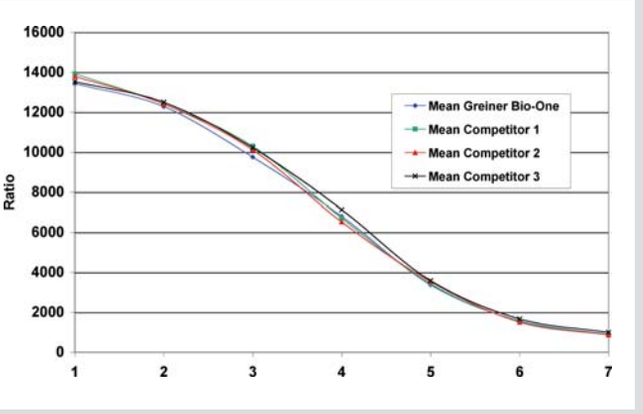
In accordance with assay protocols, a negative control (cryptate blank) was included in the test. This control contains all test components except the IP1-d2 (**Fig. 1**). Secondly, a control with unstimulated cells was used. This control consists of all the components except the ligand resulting in a maximal signal (**Fig. 5, 6, 7**).

The negative control and the control with unstimulated cells gave the expected signal values in all microplates. The highest signal with non stimulated cells was obtained in microplates from supplier 2. The lowest signal was obtained in microplates from Greiner Bio-One (**Fig. 5**). However microplates from Greiner Bio-One also exhibited the lowest standard deviation. Due to the ratiometric nature of the readout, the differences in signal strength were compensated for and the controls showed in all microplates homogenous results with low and comparable CVs (**Fig. 7**).

Obviously, the background of the plates had no detectable influence on the signal strength of the controls as it was not possible to link higher background level with a higher readout or higher variation. Therefore first indications that suggested that high background signals may reduce the final data quality of the controls or the assay were rejected. Finally, the internal controls of the IP-One HTRF assay facilitate good performance and monitoring of the assay and the data quality.

	Mean at 665 nm	CV at 665 nm	Mean at 620 nm	CV at 620 nm
Greiner Bio-One	543	5.1	659	5.1
Competitor 1	348	13.1	530	16.1
Competitor 2	598	10.4	868	11.7
Competitor 3	1337	3.4	1455	3.1

Table 1: Measurement (RFU) of empty microplates at standard HTRF conditions



Standard No.	1	2	3	4	5	6	7
IP1 final concentration in the assay [nM]	0	29	115	459	1833	7333	29333

Figure 4: Standard curves of defined IP1 concentrations established in microplates from different suppliers

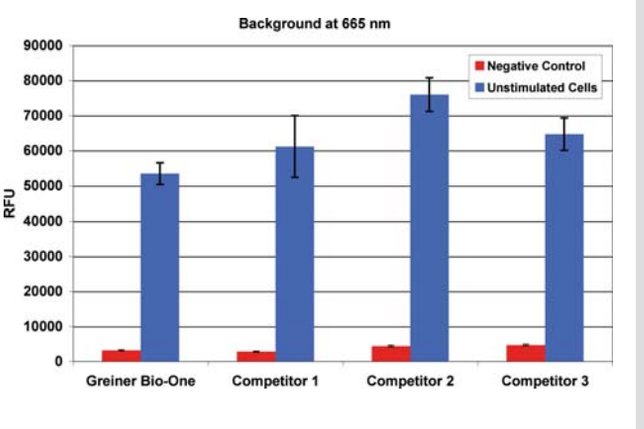


Figure 5: Background at 665 nm / Negative control and unstimulated cells

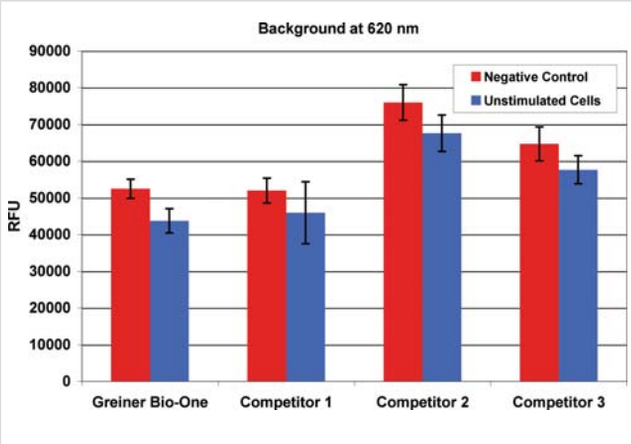


Figure 6: Background at 620 nm / Negative control and unstimulated cells

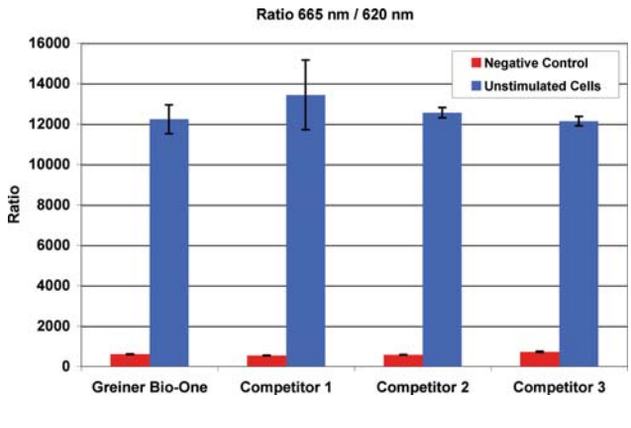


Figure 7: Signal ratio at 665 nm and 620 nm / Negative control and unstimulated cells

Assay results

The main aim of our experiment was to test the IP-One HTRF assay under standard high-throughput screening conditions. To simulate these conditions a ligand concentration of 92.59 µM was defined as a standard compound. This defined ligand concentration was tested in repetitions in randomly distributed wells in each microplate.

The signal variation in the microplates from the different suppliers was unexpectedly high (**Fig. 8, 9**). This result was surprising as the homogenous readouts of all control results suggested a homogenous assay readout as well.

The microplates from competitor 2 and 3 showed high ratio signals in the range of 7000 to 8000. But the high signal strength is linked to high standard deviation and extremely high coefficient of variation.

In contrast, the signal ratios in microplates from competitor 1 and Greiner Bio-One were in the range of 5000 but also demonstrated much lower CVs and standard deviations resulting in Z factor for the assay of 0.77 for competitor 1 and 0.80 for the plates from Greiner Bio-One.

This unexpected result of the random well testing was in accordance with the instability of the dose response curves which were created in each microplate (**Fig. 10**).

Especially microplates from competitor 2 showed higher variation or no stimulation especially at lower ligand concentrations. A dose response trend can be only assumed. However, dose response correlation in plates from Greiner Bio-One and competitor 1 was clearly visible.

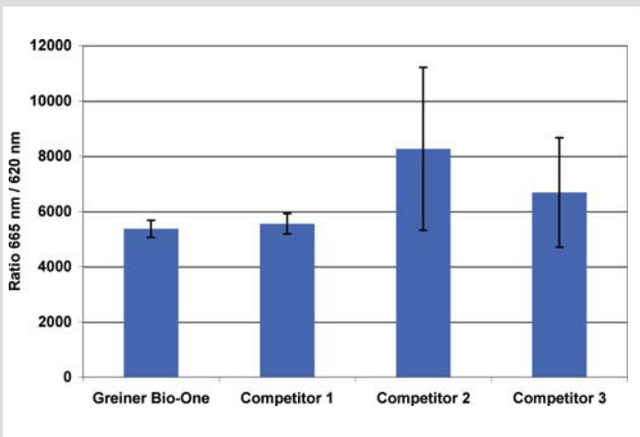


Figure 8: Signal ratio at 665 nm and 620 nm obtained with 92.59 µM stimulated cells in microplates from different suppliers.

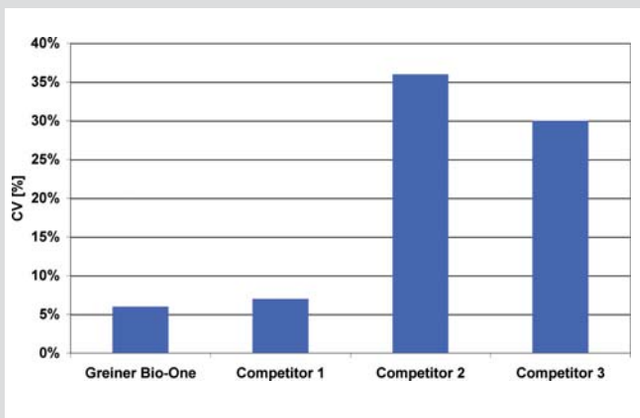


Figure 9: Coefficient of variation of signal obtained with 92.59 µM stimulated cells in microplates from different suppliers.

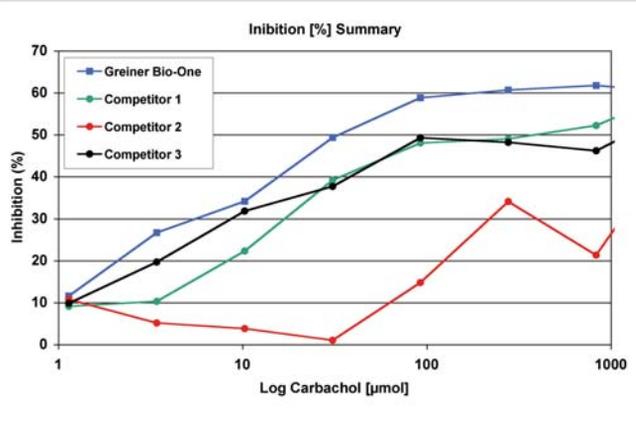


Figure 10: Dose response relationship ligand / inhibiton obtained in microplates from different suppliers.

As the controls worked perfectly in all tested microplates and did not show any significant abnormality, the only explanation for our results is that either cell growth in plates from supplier two and three had been unsatisfactory or the cells showed less vitality. With decreased vitality, receptor stimulation was either absent or not measurable especially at lower ligand concentrations.

Pipetting errors are unlikely as all microplates were treated the same way and the replicates for the different ligand concentrations did not show any obvious skips.

Conclusion

The IP-One HTRF assay has been proven as a stable cell culture time-resolved fluorescence assay for screening G-Protein coupled receptors. Thanks to clear user instructions provided by the manufacturer the assay was easy to establish.

But as an assay based on growth of adherent cells the data quality obtained is dependent upon the cell growth, the cell vitality and predisposition of the relevant receptor. As the growth of adherent cells is significantly influenced by the surface substrate upon which they grow, the quality of the microplate used appears to have a considerable effect on the quality of the data obtained.

Acknowledgement

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Germany (Main office)
Greiner Bio-One GmbH
Phone: (+49) 7022 948-0
E-Mail: info@de.gbo.com

Austria
Greiner Bio-One GmbH
Phone: (+43) 7583 6791-0
E-Mail: office@at.gbo.com

Belgium
Greiner Bio-One N. V.
Phone: (+32) 2-4 61 09 10
E-Mail: info@be.gbo.com

Netherlands
Greiner Bio-One B. V.
Phone: (+31) 172-42 09 00
E-Mail: info@nl.gbo.com

France
Greiner Bio-One SAS
Phone: (+33) 169-86 25 50
E-Mail: infos@fr.gbo.com

UK
Greiner Bio-One Ltd.
Phone: (+44) 1453-82 52 55
E-Mail: info@uk.gbo.com

Japan
Greiner Bio-One Co. Ltd.
Phone: (+81) 3-35 05-88 75
E-Mail: info@jp.gbo.com

USA
Greiner Bio-One North America Inc.
Phone: (+1) 704-261-78 00
E-Mail: info@us.gbo.com

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