Mithras LB 940 Multimode Plate Reader Used for AlphaScreen™ Signal Detection: Measurement of cAMP Production Induced by a G-protein Coupled Receptor

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Introduction

BERTHOLD TECHNOLOGIES offers a multimode plate reader, which may be equipped with a laser diode allowing the use of the Amplified Luminescent Proximity Homogeneous Assay (Alpha).

AlphaScreen™ is a bead-based non-radioactive method and can be used for measuring cAMP. cAMP acts as a second messenger for the transmission of ligand-receptor signals from the plasma membrane to intracellular proteins. Stimulation of G-protein coupled receptors may either result in an increase or decrease of cAMP production depending on the particular G-protein involved. Gs-coupled receptors, which will be exemplified here in the form of the glucose-dependent insulinotropic polypeptide receptor (GIP-R), induce cAMP production, whereas G_i-coupled receptors promote a reduction in cAMP.

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AlphaScreen™ Principle

The AlphaScreen™ technology is in principle a competition assay. The production of intracellular cAMP will generate a competition between unlabeled cAMP and exogenously added biotinylated cAMP for anti-cAMP antibodies conjugated to a bead (Acceptor bead). A streptavidin coated Donor bead will recognize the biotinylated cAMP bead complex forming a stable unit where the two beads are brought into close proximity. Upon laser excitation at 680 nm, a photosensitizer in the Donor bead converts ambient oxygen to a more excited singlet state. This step is responsible for very high signal amplification. If the beads are in close proximity, energy will be translocated to the acceptor bead and a cascade of chemical reactions will finally lead to emission of fluorescent light at 520-620 nm. A decrease in signal is observed with an increase in intracellular cAMP production and in the absence of intracellular cAMP a maximal signal is detected.

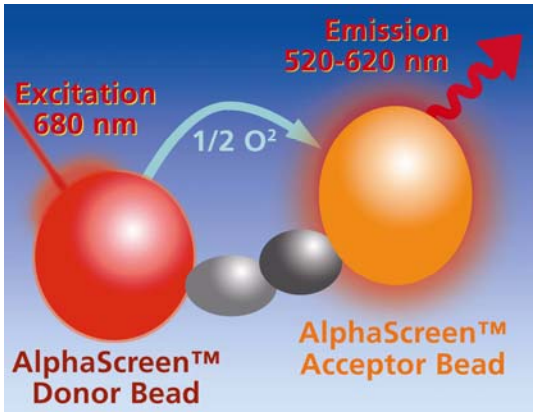

Figure 1: AlphaScreen™ principle

Experimental Procedure

Assay protocol

A stable CHO-GIP-R cell line was grown to 80% confluence at the day of experimentation. Cells were collected and re-suspended in stimulation buffer to a concentration of 1 x 10^6 cells/ml, resulting in a final amount of 15,000 cells/well. The assay was performed in 384-well microplates (OptiPlate384, PerkinElmer) and the individual assay steps are outlined below using the cAMP AlphaScreen™ kit from PerkinElmer.

Stimulation with agonist and/or antagonist

Agonist dose response curve

- Add 15 µl of cell suspension
- Add 10 µl of agonist serial dilution
- Incubate 60 min

Antagonist dose response curve

- Add 15 µl of cell suspension
- Add 5 µl antagonist serial dilution
- Incubate 15 min
- Add 5 µl of agonist
- Incubate 60 min

Addition of beads and biotinylated cAMP

Before the addition of beads, the biotinylated cAMP was pre-incubated with donor beads for 30 min in lysis buffer.

- Add 5 µl Acceptor beads (final 10.7 µg/ml)
- Incubate 10 min
- Add 5 µl of Donor beads (final 14.3 µg/ml) & biotinylated-cAMP (final 2.38 nM)
- Incubate for 1 hr (may incubate over night)

Read the plate on a Mithras LB 940 equipped with the AlphaScreen™ reading technology.

Instrument settings

Mithras LB 940 is operated through the Windows® PC software MikroWin 2000 which may also serve as a data evaluation tool. With the standard configuration pre-set parameter files for AlphaScreen™ readings are supplied which may be modified according to individual demands.

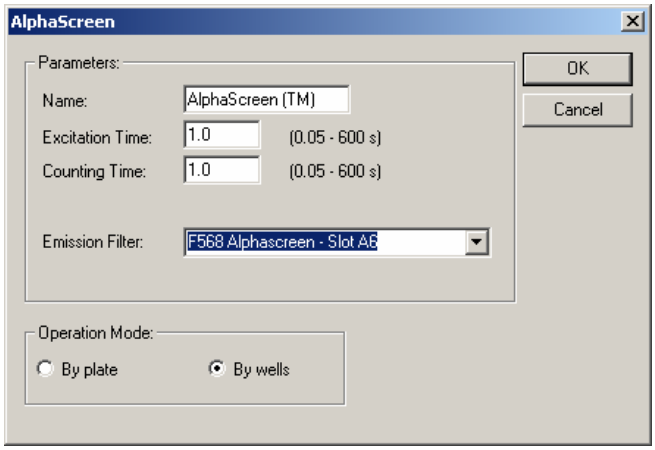

Fig.2: AlphaScreen™ operation dialogue

In order to obtain best results it is mandatory to have the correct microplate selected in the software (figure 3). The laser beam to excite the beads must be focused to the center of the wells. As microplate dimension vary between manufacturers make sure the dimensions are set correctly in the plate editor (figure 4). These settings may be checked and edited in the Plate Editor.

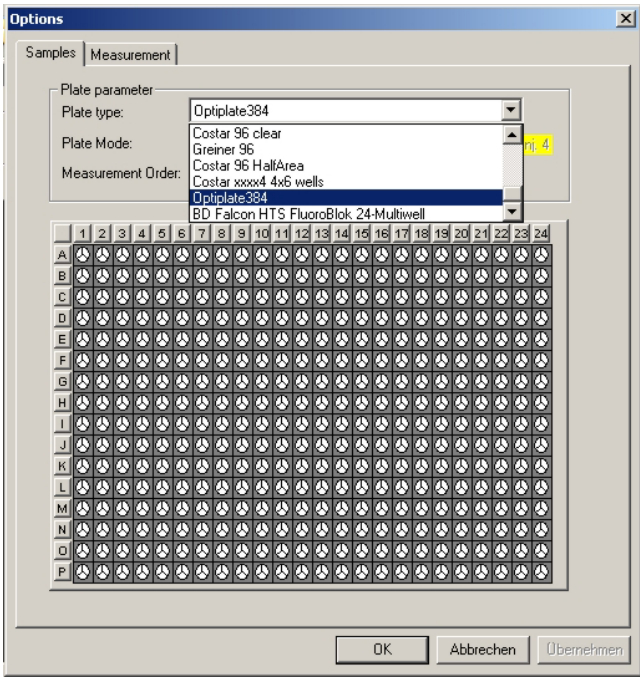

Fig.3: Selection of microplates in the Options/Read/Settings dialogue

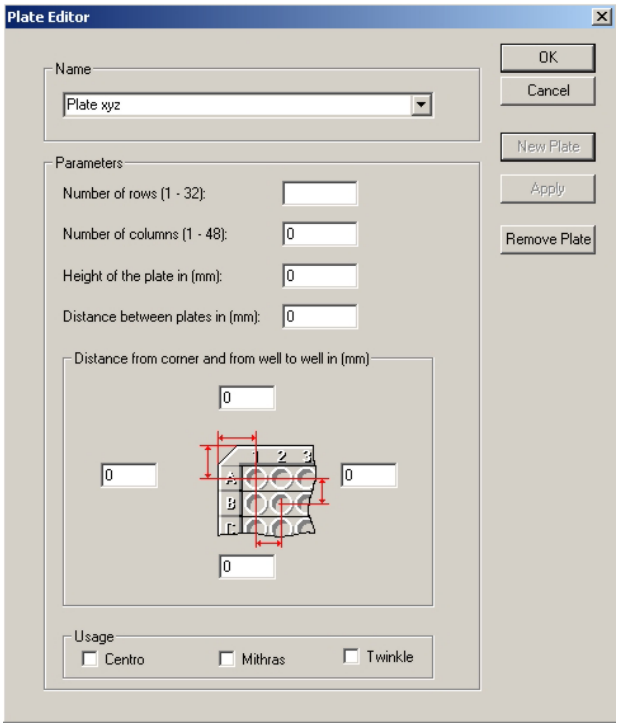

Fig.4: Editing and checking of microplate formats in the Instrument/Plate Editor

Optimal time settings for excitation and emission signals

The AlphaScreen™ signal generated by addition of buffer (maximum signal) or 1 µM of cAMP (maximum inhibition of signal) for a series of different excitation and emission times were evaluated and the stimulation-signal to background-signal ratio was calculated (Table 1). Excellent stimulation-signal to background-signal ratio was seen for all excitation/emission settings. However, the size of the signal window increased with increasing excitation and emission times. 1.0 s excitation and 1.0 s emission times were selected for the remaining experiments.

Excitation	Emission	Stimulatory signal	Background signal	Stimulatory/Background ratio
sec	sec	cps	cps	Factor
0.3	0.3	1920	29	66x
0.3	0.7	3267	66	50x
0.7	0.7	6326	94	67x
0.9	0.9	8209	115	71x
1.0	1.0	8974	136	66x
1.1	1.1	11473	149	77x
1.2	1.2	12334	173	71x

Table 1: variation of excitation and reading times

Assay optimization

A cAMP standard curve performed without cells and three dose response curves for the Forskolin-induced cAMP production using 5,000, 15,000 or 25,000 CHO-GIP-R cells were generated (Fig. 3). The cAMP concentration was measured in AlphaScreen™ counts per second. 15,000 cells per well gave the most favorable AlphaScreen™ counts, since most of the dose response curve fell within the linear region of the cAMP standard curve.

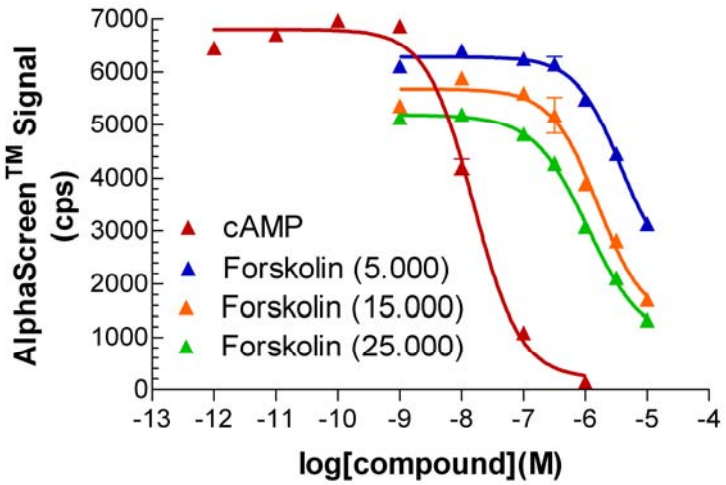

Figure 2: Dose response curve of Forskolin stimulated CHO cells

Results

Agonist and antagonist dose response curves

Dose response curves for the GIP-R agonist GIP and the GIP-R antagonist GIP(6-30)amide were performed (Fig. 2). Increasing concentrations of agonist or antagonist were added to CHO cells expressing the GIP-R. The GIP dose-response curve could be fitted with an EC50-value of 14 pM and GIP(3-30)amide inhibited an applied GIP concentration of 0.1 nM with an EC50-value of 117 nM.

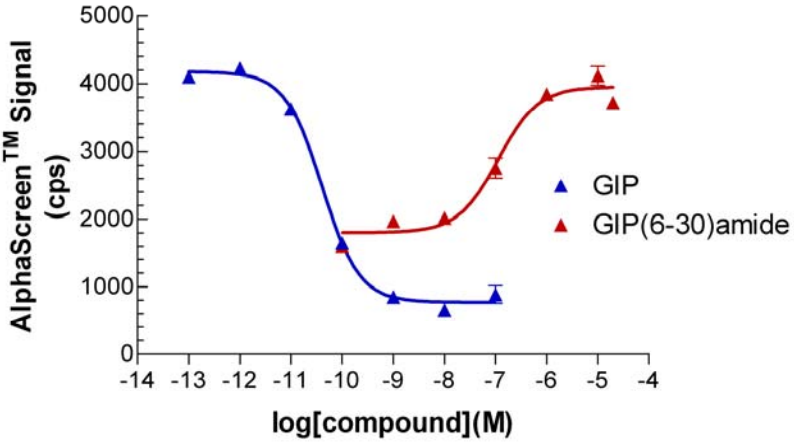

Figure 3: Dose response curve of agonist GIP and antagonist GIP(6-30)amide

Z'-value calculated for a screen performed in agonist mode

The Z'-value, which relies on both precision and assay window, was calculated to evaluate assay performance. Z'-values greater than 0.4 indicates assays, which are robust and readily transferable from assay development to screening. A Z'-value for the performance of a potential agonist screen was determined (Fig. 5). A GIP concentration of 1 nM was chosen to obtain full inhibition of the AlphaScreen™ signal. GIP was applied in wells with even column numbers and buffer was applied in wells with odd column numbers (Fig. 6). A Z'-value of 0.70 was calculated using the averages of agonist stimulated and non-stimulated signals.

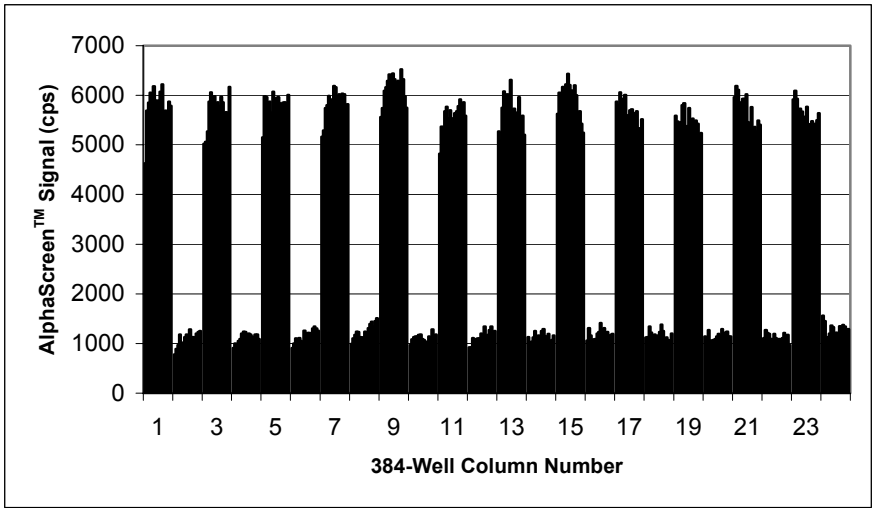

Figure 4: Agonist screen

Z'-value calculated for a screen performed in antagonist mode

For a potential antagonist screen a GIP concentration of 0.1 nM was chosen. This concentration corresponds to an inhibition of the AlphaScreen™ signal in the vicinity of the EC80-value, which often is the agonist concentration selected when screening for antagonists. GIP and buffer were applied in wells with even and odd column numbers, respectively. A Z'-value of 0.52 was obtained (Fig. 6).

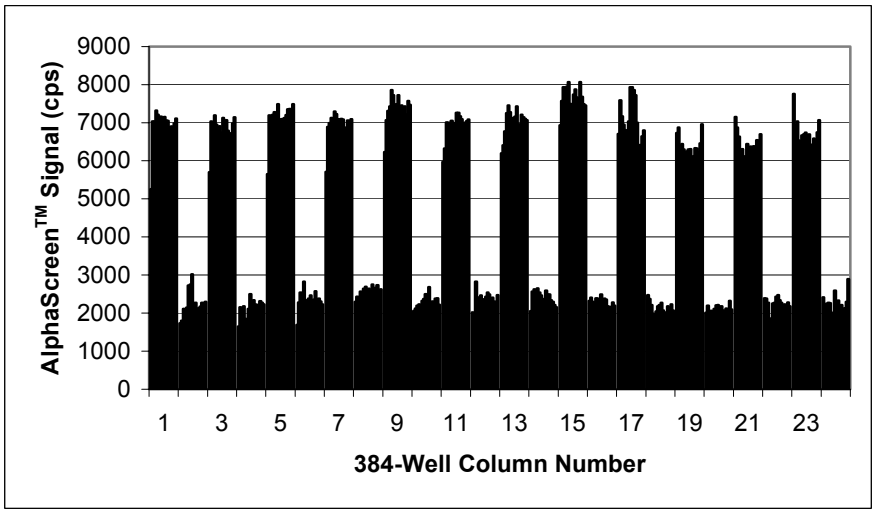

Figure 5: Antagonist screen

Conclusion

The secondary messenger cAMP, which often is the preferred readout for the response of ligands to G_s coupled GPCRs, was measured using the AlphaScreen™ technology and the performance of the assay was read by the Mithras LB 940 equipped the AlphaScreen™ reading technology. An excellent ratio of stimulation signal to background signal was seen for the control cAMP standard curve and a screening assay for the GIP-R could be developed with Z'-values above 0.5. The Mithras LB 940 not only has the capacity to be used for the AlphaScreen™ technology in a screening setting, but has also proven its worth for the development of functional BRET screening assays, as it contains up to 4 reagent injectors and is equipped with a unique optical design for bioluminescence (DOPS – Dedicated Optical Path System). Moreover, this instrument may also read luminescence, fluorescence (top and bottom reading), absorbance and fluorescence polarization.