APPLICATION NOTE

Fast and easy calcium flux assays on the VICTOR Nivo multimode plate reader.

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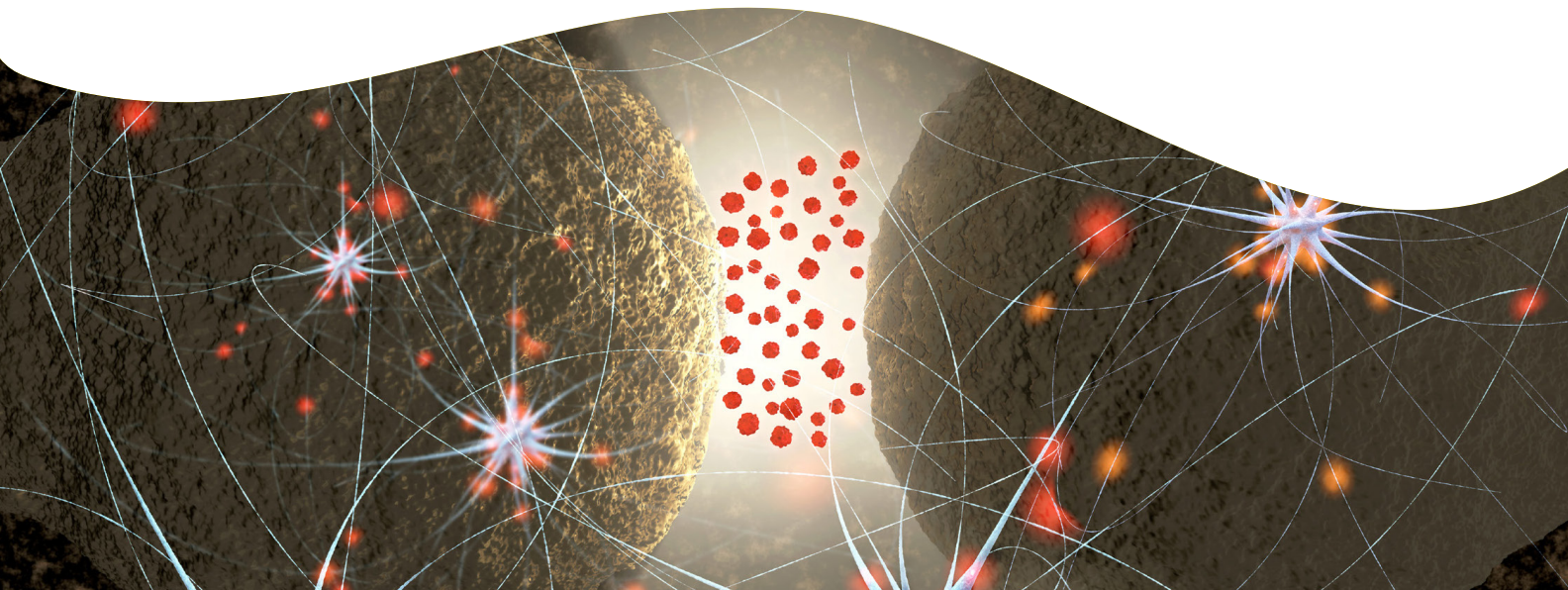
Introduction

G-Protein Coupled Receptors (GPCRs) are transmembrane proteins that are involved in many cellular signaling pathways. GPCRs now represent the target of more than 45% of all existing drugs[1].

An essential component in the study of GPCRs is the determination of their cellular activity. Changes in intracellular Ca^{2+} concentration represent a messenger for GPCR (primarily Gq/11 subtypes) signaling and allow detection of downstream effects of receptor activation. Ca^{2+} regulate directly or indirectly numerous processes including gene expression, cell motility and contraction. Various proteins such as protein kinase C (PKC), transcription factor NFAT or Calcineurin, a calcium and calmodulin dependent serine/threonine protein phosphatase, are affected by changes in $Ca2^{2+}$ concentration.[2]

In this application note we analyze fast intracellular Ca^{2+} signaling upon GPCR stimulation and inhibition using an Aequorin-bioluminescence assay measured on the VICTOR™ Nivo multimode plate reader.



Assay principle

This assay is based on the activation of the GPCR which results in cytoplasmic Ca^{2+} concentration increase: upon agonist activation of the GPCR, the $G\alpha_q$ subunit dissociates from the G-protein and induces an increase in the inositol trisphosphate concentration. This initiates the influx of calcium from the endoplasmic reticulum. The assay was performed using a CHO cell line stably expressing the human Histamine H1 GPCR and apoaequorin. Known agonists and antagonists were applied for receptor stimulation. In this type of assay, cells are incubated with Coelenterazine-h, as the co-factor of the aequorin protein. Coelenterazine-h penetrates the cell membrane and associates with the highly expressed apoaequorin protein in the presence of oxygen, leading to the reconstitution of the aequorin photoprotein. Making use of three Ca^{2+} ions, the aequorin molecule oxidizes the Coelenterazine-h substrate to Coelenteramide, releasing carbon dioxide and producing a flash of light with a peak at a wavelength of 469 nm, which is detected by the VICTOR Nivo reader.

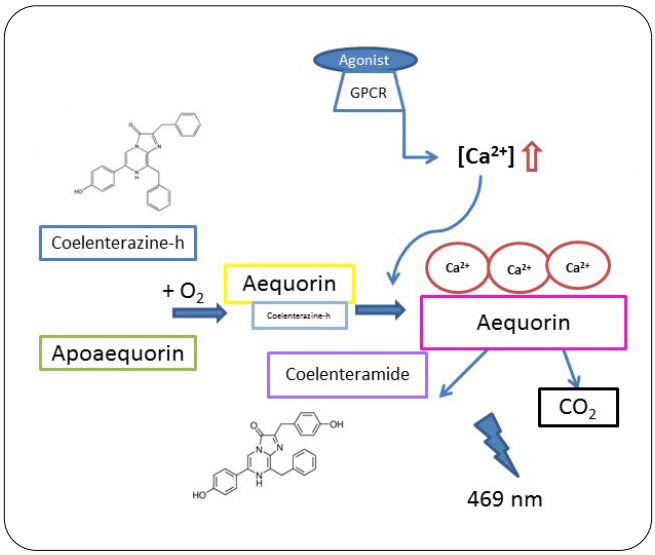


Figure 1: Assay principle. The aequorin protein is reconstituted during the cells' incubation with Coelenterazine-h. Upon receptor stimulation, the released Ca^{2+} binds to the aequorin protein, which oxidizes Coelenterazine-h to produce a flash of light at 469 nm.

The assay requires an extremely fast luminescence measurement, as the increase in intracellular Ca^{2+} concentration upon GPCR activation, and the corresponding flash of light generated, is very fast. The dispenser of the VICTOR Nivo reader meets this requirement since it can measure the light 0.1 seconds after injection, with one or two pumps, when detecting from the bottom of the microplate. There are pre-set detection protocols which can be easily adapted, if necessary, and enable single point measurements as well as kinetic detection of the luminescence signal.

Materials and methods

Cell suspension preparation

The assay was performed using the human Histamine H1 Receptor Aequorin cell line (Revvity #ES-390-A). The cells were washed with PBS (Capricorn #PBS-1A), harvested with Trypsin (Capricorn #Try-1B), resuspended in assay medium containing D-MEM/F-12 (Gibco #11039021), 0.1% BSA (Revvity #FP1322) and 5 µM Coelenterazine-h (Promega #S2011) at a concentration of 500,000 cells/mL, and incubated at room temperature (~ 25°C) on a shaker (800 rpm) protected from light using amber light protection tubes (Greiner Bio-One GmbH, #227 280) for four hours. The loading procedure with Coelenterazine-h should be performed at room temperature and protected from light due to its instability in aqueous solutions, especially at 37°C and containing serum (FBS), and sensitivity towards oxygen and Reactive Oxygen Species (ROS).

Agonist assay

For measuring the H1 receptor activity, Histamine (Sigma #H7250) was used as an agonist in the assay. The compound dilution series was prepared in 1% v/v DMSO (Roth #HN47.1) starting from 100 µM Histamine (in the final solution) and diluted by a factor of 1:4, then transferred in triplicate into a white 384-well clear flat bottom plate (Revvity ViewPlate-384, #6007490) using the Echo® Liquid Handling System 550 (Labcyte, Inc). After four hours incubation of the cells in assay medium, the injectors of the VICTOR Nivo system were washed with water and primed with the prepared cells in assay medium. The cell suspension was added (40 µL/well) to the Histamine in the wells, using the VICTOR Nivo dispenser, to a final concentration of 20,000 cells/well. The emitted luminescence was recorded using bottom reading of the plate, starting 0.1 seconds after the injection of the cells in 80 cycles for 0.1 seconds measurement time. Data analysis was performed using GraphPad Prism® software. In order to show the performance of the assay in a 96-well plate format, the agonist assay was performed in a white 96-well clear flat bottom plate (Revvity CellCarrier-96, #6005510). The volume of Histamine and cell suspension was adjusted to the plate

format, using 1 µL/well Histamine (v/v 1% DMSO) and 100 µL/well cells (50,000 cells/well). The protocol written for agonist mode was adapted for the 96-well plate. Data analysis was performed using GraphPad Prism® software.

Antagonist assay

Triprolidine (Sigma #T6764) was used as the H1 receptor antagonist. Triplicates of the Triprolidine dilution series in DMSO (1% in the final solution) starting from 100 µM (in the final solution) and diluted by a factor of 1:4 were transferred into a white 384-well clear flat bottom plate (Revvity ViewPlate-384, #6007490) using the Echo® Liquid Handling System 550 (Labcyte, Inc). After four hours incubation with Coelenterazine-h, cells were added (40 µL/well) to the Triprolidine solution and incubated at room temperature for 30 minutes. At the end of the incubation time, Histamine was added at the concentration corresponding to the EC_{80} value recorded in the agonist assay. The Histamine solution was prepared in cell culture medium (DMSO 0.0004% v/v) and added to the cells (5 µL/well) using the VICTOR Nivo dispenser.
The luminescence was recorded using the protocol for the agonist mode. The injectors were washed and primed accordingly. Data analysis was performed using GraphPad Prism® software.

Assay control

Digitonin (Sigma #D141) was used as a control for the assay, in order to measure the calcium signaling response independent of the H1 receptor. The Digitonin serial dilution was prepared in DMSO (1% v/v) starting at 400 µM (in the final solution) and diluted by a factor of 1:2 and transferred to a white 384-well clear flat bottom plate (Revvity ViewPlate-384, #6007490) using the Echo® Liquid Handling System 550 (Labcyte, Inc). The cell suspension was injected (resulting in 20,000 cells/well) using the VICTOR Nivo dispenser and the emitted light was recorded as a kinetic measurement with the same conditions as described in the agonist assay. Data analysis was performed using GraphPad Prism® software.

Z Factor

The assay robustness was calculated using a positive and a negative control. The positive control, Triprolidine, using the concentration corresponding to the IC_{80} determined from the antagonist assay, and the negative control DMSO (1% v/v), were transferred in 16 replicates into a white 384-well clear flat bottom plate (Revvity ViewPlate-384, #6007490) using the Echo® Liquid Handling System 550 (Labcyte, Inc).

After four hours incubation with Coelenterazine-h, cells were added (40 µL/well) to the DMSO or Triprolidine in the plate and incubated at room temperature for 30 minutes. Histamine at the concentration corresponding to the EC_{80} determined previously, was added to the cells (5 µL/well) using the VICTOR Nivo dispenser. The light was recorded using the protocol conditions of the agonist assay.
The injectors were washed and primed accordingly. The Z factor (Z') was determined following Iversen's equation[3]:

$$Z` = 1 - \left(\frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|} \right)$$

σp, σn being the Standard Deviations of the positive and negative controls and µp, µn the means of the positive and negative controls. Data analysis was performed using GraphPad Prism® software.

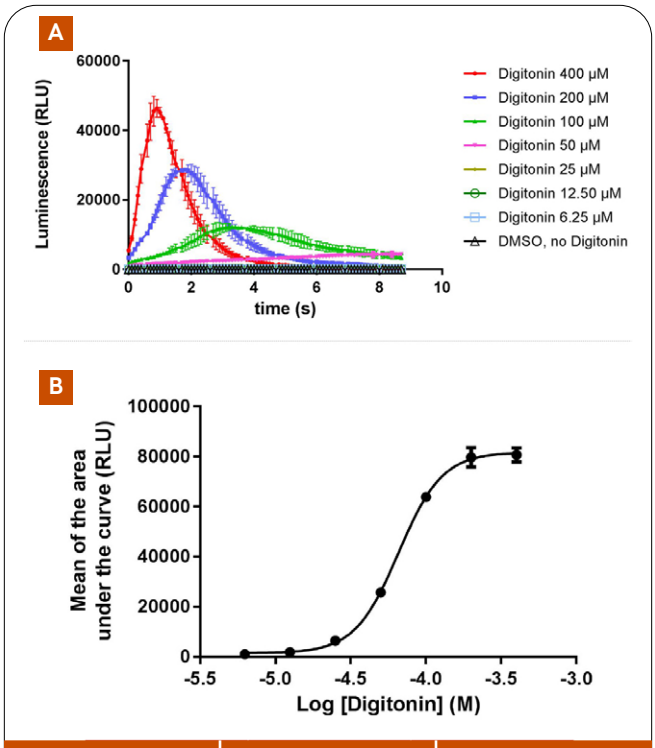
Results and discussion

Proof of principle of assay

The kinetic readout of the VICTOR Nivo system allowed the exact measurement of the luminescence signal over time after Digitonin addition (Fig. 2). It was shown that lower Digitonin concentrations generate a later light signal and the highest Digitonin concentration showed the fastest signal development. This shows the influence of the compound concentration on the time needed for the Ca^{2+} pathway to be followed and the signal to be produced. This time dependence of signal development requires a small measurement interval after cell injection to detect the full signal curve. The fast kinetic measurement of the signal curve of Digitonin by the VICTOR Nivo system improved the accuracy of detection of compound effects at each signal concentration and therefore enabled the exact calculation of the dose response curve. The signal produced in the kinetic measurements for Digitonin was integrated and the average of the area under the curve was plotted in order to generate the dose-response curve. This confirmed that Digitonin can successfully be used as an assay control in the Aequorin assay.

Cell Number titration

In order to determine the assay window for H1/Aequorin cells, a cell number titration was performed. This is important because the range of luminescence units produced by different numbers of cells per well represents varying amounts of the H1 GPCR and apoaequorin and hence different Ca^{2+} concentrations. Histamine was used as a standard agonist for the H1 receptor (Fig. 3).



Stimulator	pEC_{50} (M)	EC_{50} (M)
Digitonin	4.182	6.579×10^{-5}

Figure 2: Ca^{2+} signaling response generated GPCR-independently by Digitonin as an assay control. A: Kinetics of the Ca^{2+} signaling response generated by the assay control Digitonin. B: Dose-response curve along with the EC_{50} concentration for the GPCRindependent signal production of Digitonin.

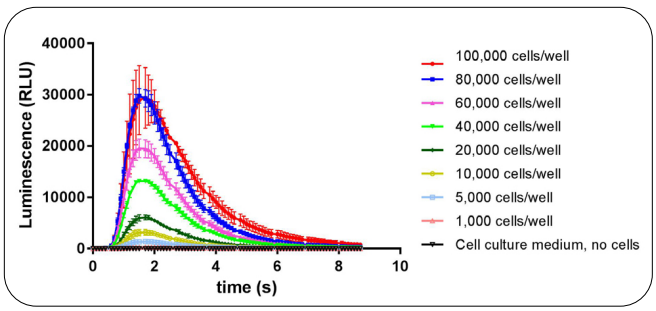


Figure 3: Luminescence signal development mediated by Histamine (1.56 µM) added to different numbers of cells per well.

The results show that the saturation of luminescence signal is reached at approx. 80,000 - 100,000 cells/well. Moreover, it was shown that the peak of the light signal is produced at the same time for all cell densities used. Therefore, 20,000 cells/well were selected to be used in further experiments, as this cell number gives a signal in the linear range of the assay and generates a reasonable signal window.

Optimization of the agonist signal detection using histamine

Using the optimized cell number, the kinetic measurements were recorded for the agonist assay upon Histamine stimulation of the H1 receptor (Fig. 4).

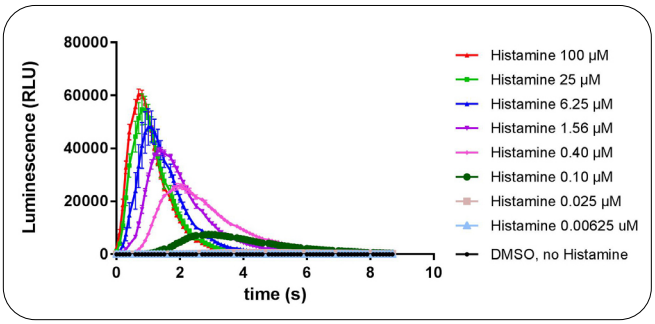
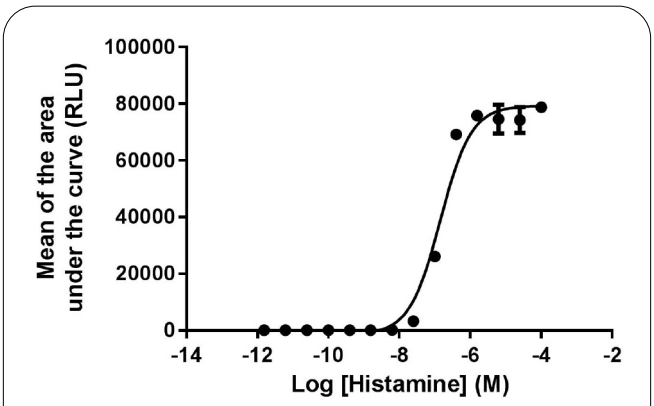


Figure 4: Kinetics of the Ca^{2+} signaling response generated by Histamine stimulation of the H1 receptor using 20,000 cells/well.

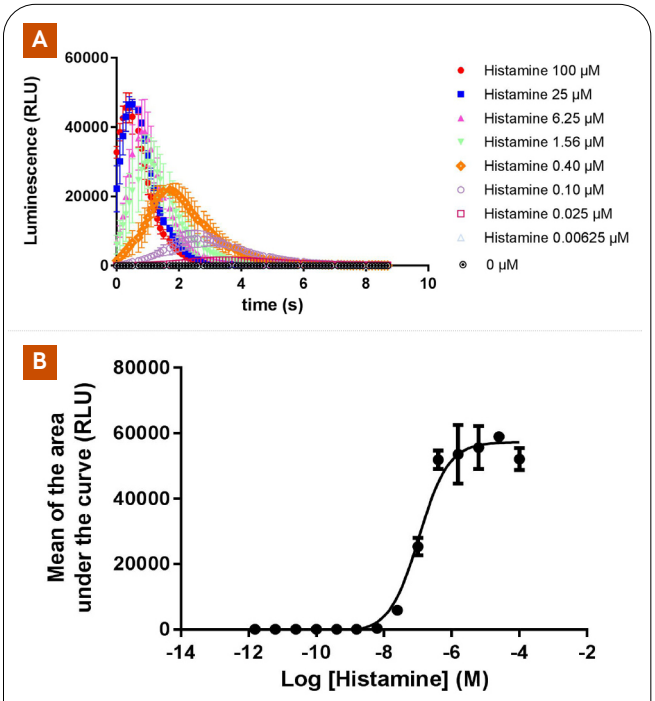
Similar to Digitonin, it was shown that lower Histamine concentrations generate a later flash of light, whereas the highest Histamine concentrations mediated the signal immediately. The signal produced in the kinetic measurements for the H1 GPCR was integrated and the average of the area under the curve was plotted in order to generate the dose-response curve. An EC_{50} of 143.4 nM was calculated for Histamine (Fig. 5). This value is in line with previously published data showing various EC_{50} for Histamine.[4,5]



Agonist	pEC_{50} (M)	EC_{50} (M)
Histamine	6.843	1.434×10^{-7}

Figure 5: Dose-response curve for stimulation of the H1 receptor by the Histamine agonist.

The flexibility of the assay was confirmed by repeating the agonist test in a 96-well plate format (Fig.6). The EC_{50} of 108 nM Histamine is in the same range as the EC_{50} of 143 nM calculated from the 384-well plate experiment, which demonstrates the suitability of the assay for both plate formats.

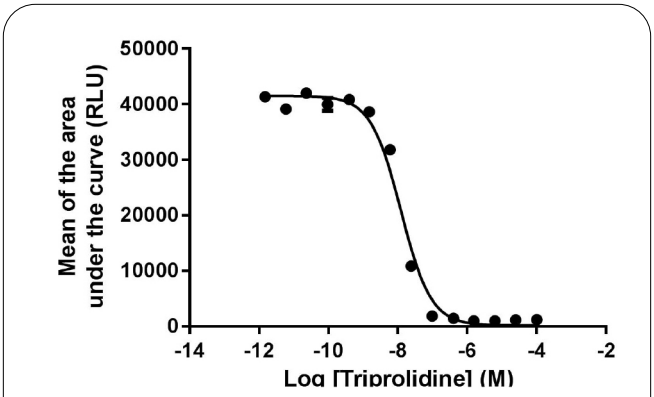


Antagonist	pEC_{50} (M)	EC_{50} (M)
Histamine	6.966	1.082×10^{-7}

Figure 6: Agonist assay for stimulation of the H1 receptor in a 96-well plate format. A: Kinetics of the Ca^{2+} signaling response generated by Histamine stimulation of the H1 receptor. B: Dose-response curve for stimulation of the H1 receptor by the Histamine agonist.

Optimization of the antagonist signal detection using triprolidine

To show the suitability of the H1-Aequorin assay for detection of receptor inhibition, the antagonist assay was performed using Triprolidine against Histamine, at the EC_{80} concentration determined in the agonist assay experiment. The assay was performed according to the protocol described in the "Materials and Methods" section. The average of the area under the curve was plotted in order to generate the dose-response curve. Fig. 7 shows the calculated dose-response of Triprolidine (IC_{50} 12.28 nM).



Antagonist	pIC_{50} (M)	IC_{50} (M)
Histamine	7.911	1.228×10^{-8}

Figure 7: Dose-response curve for inhibition of the H1 receptor by the Triprolidine antagonist against the Histamine agonist used at the EC_{80} concentration.

In order to evaluate assay quality and robustness, Z′ analysis was performed. Assay robustness was calculated using a positive control (Triprolidine) and a negative control (DMSO). The mean of the area under the curve generated in the kinetic measurement of the signal was plotted and the Z` value was calculated (Fig. 8). The signal for the 16 replicates of each control show only minor variation, therefore resulting in a Z` > 0.5 which represents good robustness and indicates the assay is suitable for screening.

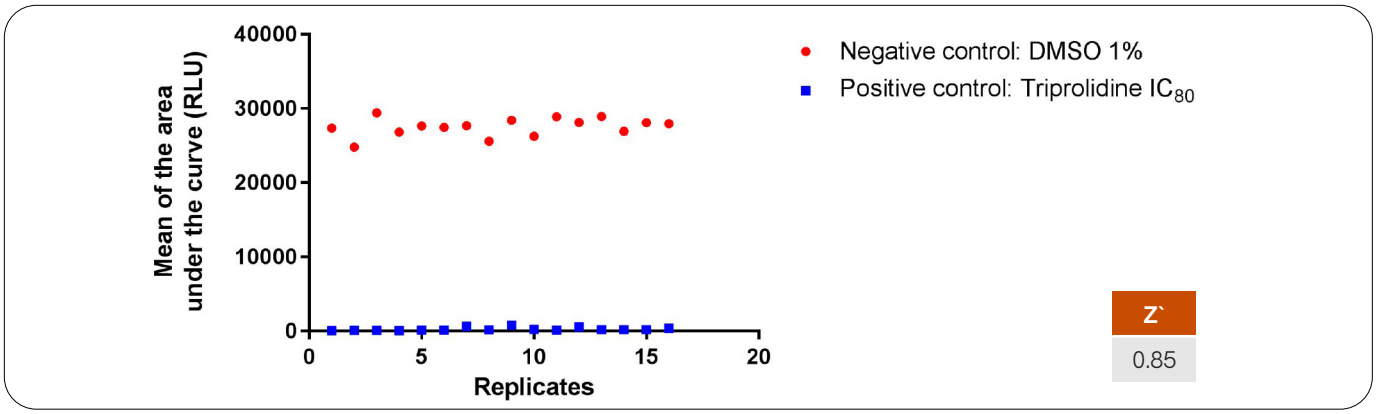


Figure 8. Aequorin assay robustness for the H1 receptor activation measurement.

Conclusion

This study describes the optimization of an Aequorin-based flash luminescence assay to detect agonist and antagonist effects on the Ca^{2+} coupled Histamine H1 GPCR. The assay demonstrates low standard deviation, low variation across the plate and accurate reproducibility. The VICTOR Nivo multimode microplate reader together with the dispenser option is well-suited to this type of assay. The simplicity and clarity of the software and the use of pre-set protocols make it easy to set up and store assay-specific measurement parameters. The use of in-well kinetic measurement of signal development and fast signal detection from the bottom of the plate in conjunction with the dispenser option of the VICTOR Nivo provides flexibility for assay optimization.

References

1. J. A. Salon, D. T. Lodowski and K. Palczewski, The significance of G-Protein Coupled Receptor Crystallography for Drug Discovery, ASPET Pharmacological Reviews (2011).

2. Berridge M.J., Lipp P., Bootman M.D. The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol. (2000).

3. Iversen P. W., Eastwood B. J., Saittampalam G. S., Cox K. A Comparison of Assay. Performance Measures in Screening Assays: Signal Window, Z´ Factor, and Assay Variability. Ratio. Biomolecular Screening (2006).

4. Oksa V. V., Kivela P., Aequorin Bioluminescence assays on MicroBeta2 LumiJet for studying Ca^{2+}-coupled GPCRs and ion channels. Application Note, Revvity (2009).

5. Technical Data sheet: human Histamine H1 Receptor Aequorin Cell Line, Revvity.

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