New Microplate Luminescence Counter for Aequorin based Ca^{2+}-coupled GPCR Assays

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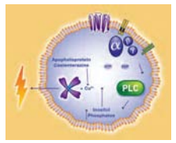
1 Introduction

The AequoScreen® technology is a generic GPCR technology based on aequorin photoprotein which can be used with G_s, G_i and G_q coupled GPCRs and calcium coupled ion channels. In the aequorin assay, cells co-expressing apoaequorin and the target receptor are first incubated with the co-factor coelenterazine in order to reconstitute the active aequorin enzyme. Reconstitution of an active aequorin, using native coelenterazine or its derivative coelenterazine h, yields an enzyme having a fast luminescent response to increasing calcium concentrations, and a high level of signal intensity. The aequorin photoprotein undergoes a bioluminescent reaction in the presence of calcium ions, producing a flash of light peaking at 469 nm. This wavelength correlates well with the maximum quantum efficiency of the PMTs used in the MicroBeta®2.

In this poster we present the Aequorin technology as performed with the MicroBeta^2 microplate counter, the latest luminescence and liquid scintillation reader from PerkinElmer, Inc. Equipped with injectors, the MicroBeta^2 LumiJET™ is shown to be uniquely suited for flash luminescence applications such as the AequoScreen technology.



AequoScreen Assay Principle
The apophotoprotein requires coelenterazine to be converted to an active aequorin enzyme. Upon calcium binding, the photoprotein oxidizes coelenterazine into coelenteraminde, producing CO_2 and emitting light. The light emission is measured as luminescence.

2 MicroBeta^2 LumiJET Microplate Counter

MicroBeta^2 is a multi-detector instrument designed for liquid scintillation or luminescence detection of samples in microplates, tubes or on filters. It is available in 1-, 2-, 6- and 12-detector versions. The 1- and 2-detector models are equipped with 24- and 96-well counting capabilities and by default the 6- and 12-detector units with 96- and 384-well support. MicroBeta^2 is available with sample capacities of 16 or 32 plates or a robot loading option.

MicroBeta^2 LumiJET can be equipped with reagent injectors needed to perform aequorin assays in 384-well plates. The performance of the MicroBeta^2 LumiJET is highly suitable for a wide range of radiolabel-based assays in addition to "flash and glow" type luminescence assays with up to 12 detectors. With two injectors per detector, the MicroBeta^2 LumiJET offers the ability run aequorin-based GPCR assays in a dual screen mode.

The software of MicroBeta^2 is familiar to existing MicroBeta users. Enhancements to the instrument have also improved assay performance where now 0.1 sec kinetic data points can be recorded and viewed, resulting in increased resolution and data quality. The kinetic reactions can be viewed during measurement or the data can be exported to PerkinElmer's AssayPro data analysis package or to other suitable software.

3 Materials and Methods

To test performance of aequorin/Ca^{2+} measurements in MicroBeta2 LumiJET, agonist and antagonist assays in two cell lines were studied. AequoScreen and AequoZen™ Frozen cells stably expressing both mitochondria-targeted aequorin and a target GPCR were used in this study as described below.

Measurements were performed with Histamine H_1 AequoScreen (PerkinElmer #ES-390-A) and Muscarinic M_5 AequoZen FroZen cell lines (PerkinElmer #ES-214-AF). The cell density in 384-well format measurements were 5,000 or 6,000 cells per well for Histamine H_1 and Muscarinic M_5 assays, respectively. Cell harvesting, coelenterazine h (Invitrogen, #C 6780) loading and preparation were done according to instructions presented in the AequoScreen Starter Kit Manual (PerkinElmer). Compound concentration series (20 µL/well) were diluted in 0.1 % BSA (Intergen, 3440-75) containing assay buffer (D-MEM/F-12, Invitrogen #11039) and prepared in white opaque OptiPlate™ 384-well microplates (PerkinElmer, #6007290). The cell suspension was dispensed on the ligands using MicroBeta^2 LumiJET (fig. 2). Injector tubing was rinsed (ethanol, water and assay buffer) and primed before injections.

Detection of agonist

Histamine (Sigma, #H7250) and acetylcholine chloride (BioChemika, #A2661) were used as an agonist for the Histamine H_1 and Muscarinic M_5 cell lines, respectively. The concentrations and dilutions series having eight replicates were prepared as instructed in the AequoScreen Starter Kit Manual. Emitted light was recorded kinetically (0.1 s measurement time) and integrated for 25 seconds.

Detection of antagonist

For the antagonist assay, cells were injected (30 µL) into the assay plate with antagonists (20 µL) using the MicroBeta^2 LumiJET. The antagonist dilution series with eight replicates was prepared as instructed in the AequoScreen Starter Kit Manual. Antagonist used for the Histamine H_1 cell line was *trans*-triprolidine (Sigma, T6764) and N-Me-Scopolamine (S8502, Sigma) for the Muscarinic M_5 cell line. Agonist (histamine or acetylcholine) at a single concentration was injected (20 µL, final concentration 10 x EC_{50}) on the preincubated (50–55min) of cells + antagonist and the emitted light was recorded kinetically (0.1 s measurement time) and integrated for 25 seconds.

Positive controls

Triton® X-100 (Fluka, #93420) at a 0.1% concentration in assay buffer was used to measure the receptor-independent cellular calcium response (cell membrane permeabilization) and acted as a positive control for the coelenterazine cell loading. Also ATP at 10 µM (ATPlite™ ATP standard, PerkinElmer #6016736) was used as a positive control for the endogenous response within CHO-K1 cells (purinergic P2Y receptor).

Determination of Z' factor

To evaluate the Z' factor for different aequorin assays, a separate Z' prime plate was prepared containing assay buffer, agonist and antagonist wells (in 60 replicates). The concentrations for agonists and antagonists in wells were 10 x EC_{50}/IC_{50}. Cells were injected into wells with MicroBeta^2 LumiJET, incubated for ~1 h and agonist (10 x EC_{50} concentrations) was added using the second injector module of MicroBeta^2 LumiJET.

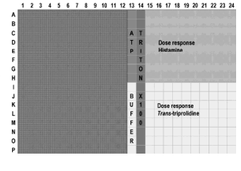
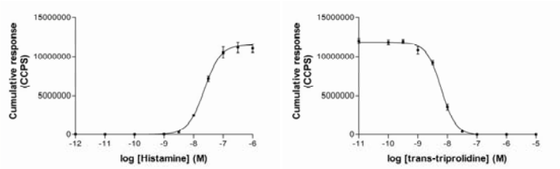


Figure 2: The plate map used for the histamine H_1 AequoScreen cell line dose response assay. Dual screen mode in MicroBeta^2 LumiJET enables to acquire data from both agonist and antagonist responses.

4 Dose Response Assays with Histamine H_1 AequoScreen Cell Line

The agonist and antagonist assay dose response curves (fig. 3, 4) demonstrate the quality of data produced by the MicroBeta^2 LumiJET. Both the EC_{50} and IC_{50} values are in accordance with previously defined values and the Z' > 0.6 for shows that AequoScreen assays can be run successfully with 384-well format using MicroBeta^2 LumiJET.



Agonist	pEC_{50}	Triton X-100 (CCPS)	S:B	% Triton X-100 response
Histamine	7.61	11900350	335	93

Z'	0.60

Figure 3: Activation of H_1 receptor by histamine. Curve fitting, error bars (SD) plotted with GraphPad Prism®.

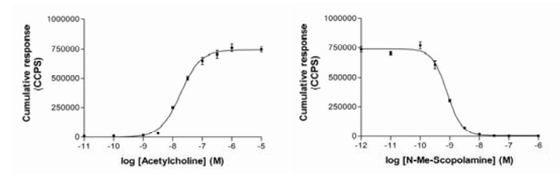
Antagonist	pIC_{50}
trans-triprolidine	8.24

Z'	0.75

Figure 4: Inhibition of H1 receptor by *trans*-triprolidine. Curve fitting, error bars (SD) plotted with GraphPad Prism.

5 Dose Response Assays with Muscarinic M_5 AequoZen FroZen Cell Line

Both the agonist and antagonist for Muscarinic M_5 receptor performed in dose dependent manner. The activation and inhibition assays produced data with high Z' values and accurate EC_{50} and IC_{50} values despite the use of a frozen cell line exhibiting low signal level.



Agonist	pEC_{50}	Triton X-100 (CCPS)	S:B	% Triton X-100 response
Acetylcholine	7.72	1179899	65	64

Z'	0.53

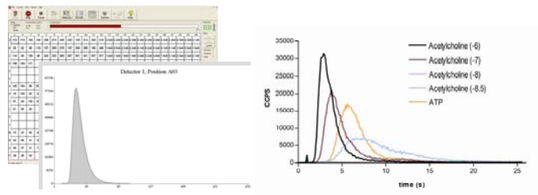
Figure 5: The agonist dose-response curve for acetylcholine on M_5 Muscarinic FroZen cell line. Curve fitting, error bars (SD) plotted with GraphPad Prism.

Antagonist	pIC_{50}	S:B
trans-triprolidine	9.1	195

Z'	0.72

Figure 6: N-Me-Scopolamine inhibited the stimulation of M_5 receptor by acetylcholine. Curve fitting, error bars (SD) plotted with GraphPad Prism.

6 Kinetic Mode



Kinetic data can be observed is MicroBeta^2 instrument software's Live view in numeric and graphical representations as the data is collected. Data can also be exported in text files and analyzed in data analysis software (the Muscarinic M_5 antagonist response curves as plotted with GraphPad Prism, shown right).

7 Evaluating the Working Cell Density

Cell suspension assay was evaluated at a range of cell densities (500 – 15000 cells/well). At cell density of 5000 and over, the assay is very robust as the Z' value is >0.7. Even at lower cell numbers the assays still shows a good performance.

Cell density (cells/well)	Z'	S:B
15 000	0.76	1157
5 000	0.71	401
2 500	0.59	137
1 000	0.38	115
500	0.43	70

These assays were conducted by pre-dispensing Histamine H_1 AequoScreen cells (prepared as before) together with buffer or antagonist (*trans*-triprolidine, final concentration 10 x IC_{50}) to OptiPlate-384 in 36 replicates. The agonist (histamine, final concentration 10 x IC_{50}), was dispensed and resulting luminescence was recorded by MicroBeta^2 LumiJET (total volume, 70 µl).

8 Summary

The MicroBeta^2 LumiJET introduces new features and possibilities for a variety of assays to be run with throughput and ease. The aequorin assays that are presented here were run with a 12-detector MicroBeta^2 model with two injectors per detector. This is ideal for flash type luminescence assays as it combines high throughput with high sensitivity luminescence detection, therefore providing all tools for studying calcium related GPCR and ion channel assays. With MicroBeta^2 the GPCR assays can be run in single mode to determine agonist or antagonist effect of a molecule, or in dual screen mode to determine both agonist and antagonist response for one sample plate. In dual screen mode the plate is run sequentially, generating approximately 20 minutes effective time in a 384-well plate for a possible antagonist to interact with cell membrane targets.

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