

## 1 Introduction

The AequoScreen® technology is a generic GPCR technology based on aequorin photoprotein which can be used with G<sub>s</sub>, G<sub>i</sub> and G<sub>q</sub> coupled GPCRs and calcium coupled ion channels. In the aequorin assay, cells co-expressing aequorin and target receptor are first incubated with co-factor coelenterazine in order to reconstitute the active aequorin enzyme. Reconstitution of an active aequorin with 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 MicroBeta<sup>2</sup>.

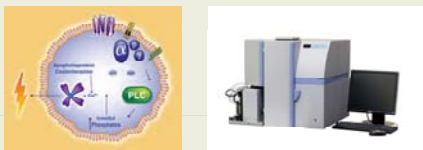


Figure 1. 2. AequoScreen Assay Principle. The apophotoprotein requires coelenterazine to be converted to an active aequorin enzyme. Upon calcium binding, the photoprotein oxidizes coelenterazine into coelenteramide, producing CO<sub>2</sub> and emitting light. The light emission is measured as luminescence with a suitable instrument e.g. MicroBeta<sup>2</sup> LumiJET.

## 2 MicroBeta<sup>2</sup> LumiJET Microplate Counter

MicroBeta<sup>2</sup> 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<sup>2</sup> is available with sample capacities of 16 or 32 plates or a robot loading option.

MicroBeta<sup>2</sup> can be equipped with reagent injectors needed to perform aequorin assays. This model is called the MicroBeta<sup>2</sup> LumiJET. The performance of the MicroBeta<sup>2</sup> LumiJET is highly suitable for a wide range of radiolabel-based assays in addition to "flash and glow" type luminescence assays. With two injectors per well, for up to 384-well plates and twelve detectors, the MicroBeta<sup>2</sup> LumiJET offers the ability to run aequorin-based GPCR assays in a dual screening mode, providing both agonist and antagonist information about a compound.

To test performance of aequorin/Ca<sup>2+</sup> measurements in MicroBeta<sup>2</sup> 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.

## 3 Materials and Methods

Measurements were performed with Histamine H<sub>1</sub> AequoScreen (PerkinElmer #ES-390-A) and Muscarinic M<sub>5</sub> AequoZen FroZen cell lines (PerkinElmer #ES-214-AF). The cell densities in 384-well plate format were 5,000 or 6,000 cells per well for Histamine H<sub>1</sub> and Muscarinic M<sub>5</sub> assays, respectively. Cell harvesting, coelenterazine h (Invitrogen, #C 6780) loading and preparation were done according to instructions presented in 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 OptiPlate™ 384-well microplates (PerkinElmer, #6007290). The cell suspension was dispensed on the ligands using MicroBeta<sup>2</sup> LumiJET. Injector tubing was rinsed (ethanol, water and assay buffer) and primed before injections.

Histamine (Sigma, #H7250) and acetylcholine chloride (BioChemika, #A2661) were used as an agonist for the Histamine H<sub>1</sub> and Muscarinic M<sub>5</sub> 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.

For the antagonist assay, cells were injected (30 µl) into the assay plate with antagonists (20 µl) using the MicroBeta<sup>2</sup> LumiJET. The antagonist dilution series with eight replicates was prepared as instructed in the AequoScreen Starter Kit Manual. Antagonist used for the Histamine H<sub>1</sub> cell line was *trans*-triprolidine (Sigma, T6764) and *N*-Me-Scopolamine (S8502, Sigma) for the Muscarinic M<sub>5</sub> cell line. Agonist (histamine or acetylcholine) at a single concentration was injected (20 µl, final concentration 10 × EC<sub>50</sub>) on the preincubated (50–55 min) cells + antagonist and the emitted light was recorded kinetically (0.1 s measurement time) and integrated for 25 seconds.

Triton® X-100 (Fluka, #93420) at 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. 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).

To evaluate the Z' factor for different aequorin assays, a separate Z' plate was prepared with assay buffer, agonist and antagonist wells (in 60 replicates). The concentrations for agonists and antagonists were 10 × EC<sub>50</sub>/IC<sub>50</sub>. Cells were injected into wells with MicroBeta<sup>2</sup> LumiJET, incubated for ~1 h and agonist (10 × EC<sub>50</sub>) was added using the second injector module of MicroBeta<sup>2</sup> LumiJET.

## 4 Dose Response Assays

The agonist and antagonist assay dose response curves demonstrate the quality of data produced by the MicroBeta<sup>2</sup> LumiJET. Both the EC<sub>50</sub> and IC<sub>50</sub> values are in accordance with previously defined values. High Z' values show that AequoScreen assays with either AequoScreen or AequoZen FroZen cell lines can be run successfully in 384-well format using MicroBeta<sup>2</sup> LumiJET.

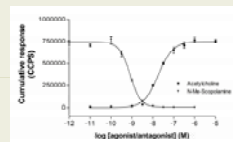


Figure 3: Activation and inhibition of M<sub>5</sub> receptor in Muscarinic M<sub>5</sub> AequoZen FroZen cell line. Acetylcholine and *N*-Me-Scopolamine were used as agonist and antagonist, respectively. Curve fitting, error bars (SD) plotted with GraphPad Prism®.

| Agonist       | pEC <sub>50</sub> | Z'   | S:B | % Triton X-100 response |
|---------------|-------------------|------|-----|-------------------------|
| Acetylcholine | 7.72              | 0.53 | 65  | 64                      |

| Antagonist                 | pIC <sub>50</sub> | S:B | Z'   |
|----------------------------|-------------------|-----|------|
| <i>trans</i> -triprolidine | 9.1               | 195 | 0.72 |

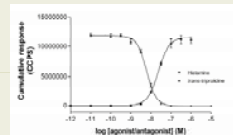


Figure 4: Activation and inhibition of H<sub>1</sub> receptor in Histamine H<sub>1</sub> AequoScreen cells. Histamine and *trans*-triprolidine were used as agonist and antagonist, respectively. Curve fitting, error bars (SD) plotted with GraphPad Prism®.

| Agonist   | pEC <sub>50</sub> | Z'   | S:B | % Triton X-100 response |
|-----------|-------------------|------|-----|-------------------------|
| Histamine | 7.61              | 0.60 | 335 | 93                      |

| Antagonist                 | pIC <sub>50</sub> | Z'   |
|----------------------------|-------------------|------|
| <i>trans</i> -triprolidine | 8.24              | 0.75 |

## 5 Kinetic Result Viewer

The software of MicroBeta<sup>2</sup> 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 better 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.

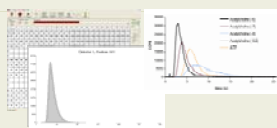


Figure 5: Kinetic views. Kinetic data represented in MicroBeta<sup>2</sup> instrument software and data from Muscarinic M<sub>5</sub> antagonist response curves as plotted with GraphPad Prism®.

## 5 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.

These assays were conducted by pre-dispensing Histamine H<sub>1</sub> AequoScreen cells (prepared as before) together with buffer or antagonist (*trans*-triprolidine, final concentration 10 × IC<sub>50</sub>) to OptiPlate-384 in 36 replicates. The agonist (histamine, final concentration 10 × EC<sub>50</sub>), was dispensed and resulting luminescence was recorded by MicroBeta<sup>2</sup> LumiJET (total volume, 70 µl).

| 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   |

## 6 Summary

The MicroBeta<sup>2</sup> 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<sup>2</sup> model with two injectors per well. 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<sup>2</sup> 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 responses 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.