# Multiplexing approach for GPCR signaling pathways in HTS format


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## Introduction

Small molecules modulating GPCR are able to selectively affect one signaling pathway over other for specific GPCR thereby "biasing" the signaling. The current explanation for this biased agonism is that GPCRs can adopt multiple active conformations stabilized by different molecules affecting intracellular signaling in different ways. This divergent behavior brings further complexity in a Drug Discovery program and thus requires a methodology improvement for providing *in vitro* pharmacology and SAR optimization in fastest way. Such improvement can be achieved by a multiplexing approach enabling intracellular signaling analysis simultaneously.GPR43 (also known as FFA2), a G-protein coupled receptor, is activated by short-chain fatty acids (SCFA) including propionate and acetate and is coupled to both $G_i$ and $G_q$ protein. GPR43 has been investigated in models pertaining to the treatment of Type 2 Diabetes and Dyslipidemia. A synthetic, small-molecule Phenylacetamide (Φacetamide) has recently been published to activate GPR43 in an allosteric manner (1, 2) and to induce an inhibition of lipolysis through a Gi intracellular coupling. These data indicate that GPR43 agonists may have an important therapeutic role in the management of type II diabetes, dyslipidemia and aspects of the metabolic syndrome.

We present here the development of a 384-well plate multiplexed assay using both Aequorin and cAMP HTRF™ for the evaluation of $G_q$- and $G_i$-coupling in response to SCFA and Φacetamide as well as identification of biased small molecule agonists.

## Material & Methods

CHO-K1 cells expressing recombinant human GPR43 receptor and mitochondrial apoaequorine were loaded with Coelenterazine h and were injected on the test compounds in a 384-well plate. The resulting emission of light was measured using a FDSS6000 luminometer (Hamamatsu).

After a 30 minutes incubation with the test compounds at room temperature, HTRF™ cAMP-d2 and anti-cAMP-cryptate conjugates were added to the cells and the cAMP levels were measured according to the manufacturer's instructions (CisBio International) on a Rubystar plate reader (BMG Labtech).

In these experiments, the different kinetics of signal transduction allowed to discriminate between the rapid response to variations in $Ca^{2+}$ flux ($G_q$ signaling – aequorin assay) and the slower response to cAMP modulation ($G_i$ signaling – cAMP HTRF™ assay).

This allowed to monitor two separate signaling pathways for the same receptor, in the same wells and to search for compounds displaying biased-agonism properties.

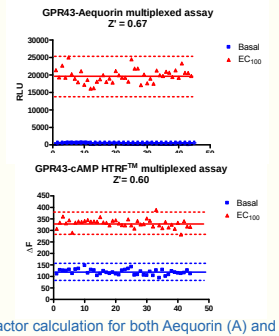


**Figure 1.** Z' factor calculation for both Aequorin (A) and cAMP HTRF™ (B) assays performed sequentially as a multiplex assay. Both assays display robust performance with Z' factors equal to or greater than 0.6

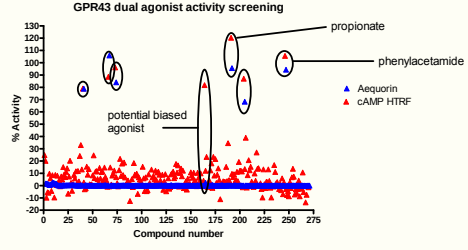


**Figure 2.** Screening of 270 compounds at 10 µM for agonist activity in recombinant human GPR43 Aequorin-cAMP HTRF™ multiplex assay. Reference compounds propionate and phenylacetamide were included as positive controls at 10 mM and 10 µM respectively. Five active compounds were selected for follow-up in dose-response testing, one of which displayed potential biased agonism properties.

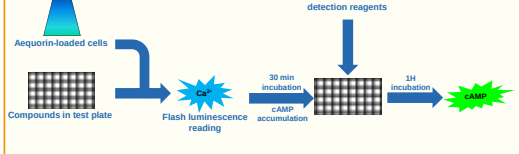


**Figure 3.** Schematic of the multiplexing procedure

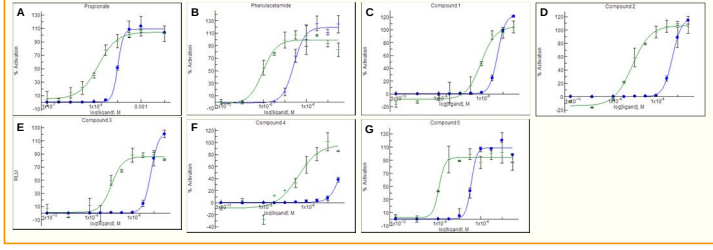


**Figure 4.** Dose-response testing following the screening at 1 concentration. Propionate and phenylacetamide reference compounds were tested along the five active compounds selected from the screening. Data are presented in % activation in Aequorin assay (blue) and in cAMP assay (green), for Propionate (A), Phenylacetamide (B), Compound1 (C), Compound 2 (D), Compound 3 (E), Compound 4 (F) and Compound 5 (G). In all cases, the $EC_{50}$ is left-shifted in the cAMP read-out but this shift is more pronounced for some of the compounds compared to the references. Individual values for all compounds in both read-outs are reported in Table 1.

**Table 1. Measured $EC_{50}$ values in both read-outs**

| Compound ID | $EC_{50}$ (nM) | | % Activity at 10 µM or 10 mM* | | Left-shift (Aeq/cAMP) |
|---|---|---|---|---|---|
| | Aeq | cAMP | Aeq | cAMP | |
| Propionate | 103000 | 14900 | 103* | 110* | 6.9 |
| Phenylacetamide | 257 | 9.06 | 111 | 97 | 28 |
| Compound1 | 5580 | 941 | 99 | 96 | 5.9 |
| Compound2 | 5800 | 76.7 | 88 | 110 | 75 |
| Compound3 | 7390 | 105 | 83 | 89 | 70 |
| Compound4 | 42200 | 410 | 7.6 | 101 | 103 |
| Compound5 | 359 | 10.8 | 120 | 102 | 33 |

## Conclusions

- We have set up assay conditions where two read-outs can be measured sequentially in the same well : Aequorin $Ca^{2+}$ flux assay ($G_q$ signaling) and cAMP HTRF™ assay ($G_i$ signaling)
- Both read-outs show good Z´ factors and so are suitable for compound high throughput screening
- We have performed a small screening looking for compounds that would present biased agonism properties.
- In a follow-up experiment, the candidate compounds were confirmed as positive hits and generally displayed a better $EC_{50}$ in cAMP assay. Three of them were significantly more shifted and represent potential biased GPR43-$G_i$ agonists.
- This methodology adds a new dimension to the hit-to-lead screening with an immediate confirmation in a secondary assay for balanced agonists and with the direct identification of new relevant entities presenting a biased efficacy for signaling to different G proteins.


References

(1) Ge H et al., Endocrinol 2008, 149:4519

(2) Wang Y et al., Bioorg. Med. Chem. 2010, 20 (2): 493