

USING LUCIFERASE REPORTER ASSAYS TO SCREEN FOR GPCR MODULATORS

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Previously, we described a strategy to construct stable cell lines expressing two luciferases on two plasmids for a GPCR reporter assay. In this report, we further demonstrate the utilities and advantages of the dual-reporter luciferase GPCR assay through additional examples including an agonist screen of a LOPAC compound collection.

Introduction

G-protein-coupled receptors (GPCRs) represent one of the most important classes of drug targets, with an estimated 50% of currently marketed medicines directed against GPCRs (1). With over 150 additional orphan GPCRs in the human genome that remain to be characterized, this target class continues to generate strong interest for drug discovery.

GPCR signaling pathways can be categorized into three classes based on the G protein α -subunit involved, G_s , G_q , and G_{10} . These subunits mediate signaling through distinct pathways that lead to the modulation of gene transcription via specific response elements (RE), which are located upstream of the regulated genes. Reporter assays have been used successfully in the process of GPCR-targeted drug discovery by coupling receptor modulation to regulation of reporter gene transcription (Figure 1).

The dual-reporter luciferase method is well suited for studying GPCR modulators in a high-throughput format.

Among various genetic reporters such as alkaline phosphatase, β -lactamase and green fluorescent protein, luciferase is preferred because of its sensitivity, dynamic range, fast and easy quantitation and lack of endogenous activity (2,3). In addition, specially developed assay reagents allow facile measurement of two luciferase activities in the same sample.

Previously, we presented a strategy for GPCR assays in which stable cell lines are constructed to express two luciferases on two plasmids (4). One plasmid expresses a destabilized firefly luciferase (e.g., Luc2P) under the control of a response element and a hygromycin selectable marker. The second plasmid expresses the target GPCR and a *Renilla* luciferase-neomycin selectable marker fusion (Figure 2). We demonstrated this concept for GPCR screening using an example of the dopamine receptor D1 (DRD1) expressed in HEK293 cells with a luciferase gene coupled to a CRE sequence (pGL4-CRE-*luc2P*). In this report we further demonstrate the utilities and advantages of the dual-reporter luciferase GPCR assay through additional examples including an agonist screen of a LOPAC compound collection.

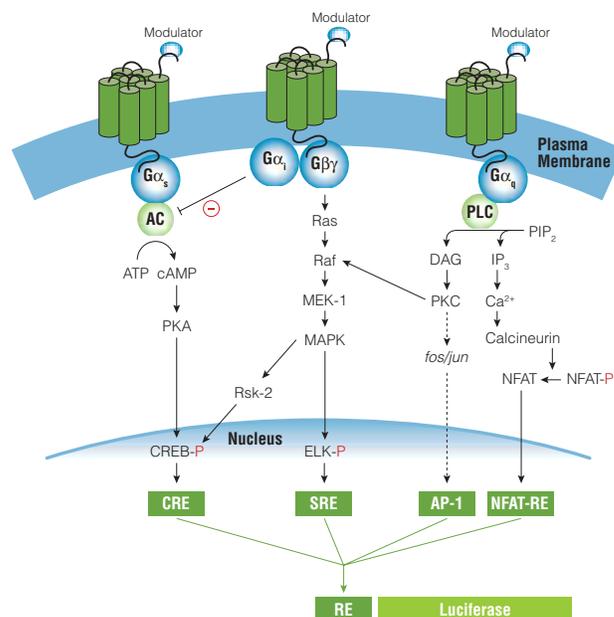


Figure 1. Schematic diagram showing GPCR signaling pathways. Upon stimulation, G_{α_s} -coupled receptors activate adenylyl cyclase (AC) resulting in an increase in cAMP; G_{α_i} -coupled receptors inhibit AC; the $\beta\gamma$ subunits activate the MAP kinase pathways; G_{α_q} -coupled receptors activate phospholipase C (PLC) to produce inositol triphosphate, which in turn increases intracellular calcium concentration. CRE, cAMP response element; SRE, serum response element; AP-1, activator protein 1, and NFAT-RE, nuclear factor of activated T-cell response element.

pGL4-RE-*luc2P*



pF9A CMV *hRluc-Neo*



Figure 2. A diagram of two plasmids involved in the dual-luciferase GPCR assay. RE, response element/promoter; *luc2P*, destabilized firefly luciferase with PEST sequence (proline, glutamate, serine, threonine); PSV40, SV40 promoter; Hygr, hygromycin resistance gene; P_{CMV}, CMV promoter; *Rluc-Neo*, *Renilla* luciferase and neomycin resistance gene fusion. PEST sequences are associated with rapidly degraded proteins. More information about the pGL4 vectors available online at: www.promega.com

Screening for GPCR Modulators with Luciferase Reporters

Table 1. High-Quality Data from Multiple Cell Lines Representing Multiple GPCR Pathways.

Response Element	Receptor	G-Protein Subunit	Fold Induction	Z'-Factor Value in 384-Well Format	Agonist
CRE	DRD1	G α_s	56	0.77	SKF38393
CRE	M3R	G α_s	18	0.70	carbachol
NFAT	M1R	G α_q	23	0.67	muscarine chloride
NFAT	M3R	G α_q	20	0.73	muscarine chloride

HEK293 cells were transfected with pGL4-RE-*luc2P* plasmid and selected for hygromycin resistance. The clones with the combination of high induction of CRE or NFAT and high luciferase expression were chosen and transfected with pF9A with the desired GPCR. Hygromycin and neomycin double-resistant clones were isolated. The best clones were selected based on induction by the receptor-specific agonist. An agonist assay was performed in a 384-well plate format to determine induction and Z'-factor value. Ten thousand cells/well were plated. Half of the wells were stimulated with agonist and half were mock stimulated. Cells were harvested after 4 hours for CRE/DRD1, 5 hours for CRE/ M3R, and 8 hours for NFAT/M3R and M1R. Luciferase activity was determined using the Dual-Glo™ Luciferase Assay System (Cat.# E2920) and quantified using the Berthold® Mithras LB 96 V or Tecan GENios Pro® luminometers. Induction was calculated as the average firefly stimulated RLU/average mock stimulated RLU.

High-Quality Assay for High-Throughput Screening

We generated two stable cell lines harboring CRE or NFAT response elements upstream of firefly luciferase. These two response elements correspond to the main signaling pathways employed by the majority of GPCRs (Figure 1). We then incorporated into these cells various receptors, including dopamine receptor DRD1 and the muscarinic receptors M1 and M3 (Table 1). The resulting doubly transfected, stable cell lines were evaluated for their performance in screening assays by determining the Z'-factor value for an agonist assay in 384-well plate format ($Z' = 1 - [(3 * SD_{high} + 3 * SD_{low}) / (average_{high} - average_{low})]$). All assays showed Z'-factor values significantly higher than 0.5, an acceptance criterion commonly used for high-throughput screening (5). In addition, the response dynamics (fold of induction) were much greater than typically reported for some of the other assay formats, allowing easier identification of partial agonists and ranking of compound potency (see below).

Identify Partial Agonists

Partial agonists are compounds that can stimulate receptor activity, but it is unclear if that receptor activity is the same that a full agonist can achieve. Since the reporter responds through an amplified signal transduction process, the assay might have difficulty distinguishing partial and full agonists

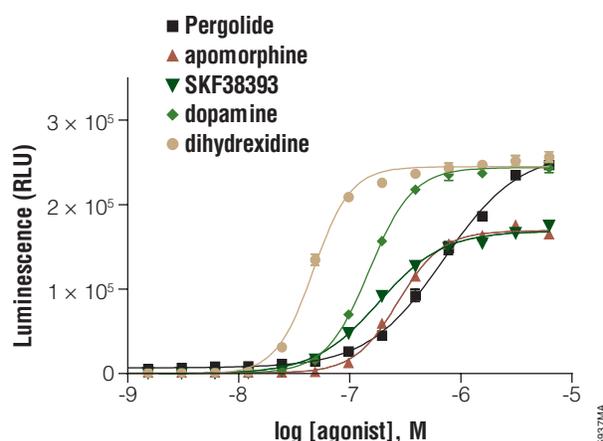


Figure 3. Detection of DRD1 partial agonists. The doubly transfected, stable CRE/DRD1 cell line was plated at 10,000 cells/well in a 96-well plate. Each agonist was serially diluted 1:2, then added to wells in replicates of 4, beginning with 50 μ M. Cells were incubated with agonist for 4 hours, harvested and analyzed using the Dual-Glo™ Assay System (Cat.# E2920). Luciferase activity was measured on the GloMax™ 96 Microplate Luminometer (Cat.# E6501).

(2). To evaluate this, we used HEK293 cells with CRE-*luc2P*/DRD1 to conduct dosage response studies with several agonists for the D1 receptor (Figure 3). SKF38393 and apomorphine showed lower induction of reporter expression compared to the other three agonists, suggesting that these compounds are partial agonists for DRD1. This conclusion is supported by studies using other methods (6).

Rank Potency of Agonists and Antagonists

In our previous article (4), we showed that this reporter assay generated biologically relevant EC₅₀ and IC₅₀ values for an agonist and an antagonist of DRD1, respectively. We expanded our evaluation to a variety of modulators using the assays shown in Table 1. Results using HEK293 with NFAT-*luc2P*/M3R as an example are shown in Figure 4 to demonstrate the capability of ranking potency of either agonists or antagonists. Among the agonists, muscarine chloride is most potent, with EC₅₀ of 8nM, followed by carbachol, pilocarpine and Mcn-A-343. Among the antagonists, scopolamine is the most potent, with IC₅₀ of 1.1nM, followed by atropine, telenzepine and pirenzepine.

Identify GPCR Agonists in LOPAC Library Compounds

To demonstrate the ability for high-throughput compound screening using this assay, we performed an agonist screen using LOPAC compounds (Sigma-Aldrich). The LOPAC collection is an annotated library of 1280 compounds from 56 pharmacological classes. The screen was performed in a 384-well format using 10 μ M of each compound. Results from a sample plate of NFAT-*luc2P*/M3R screen are shown in Figure 5. Hits were defined as reporter expression greater than 3

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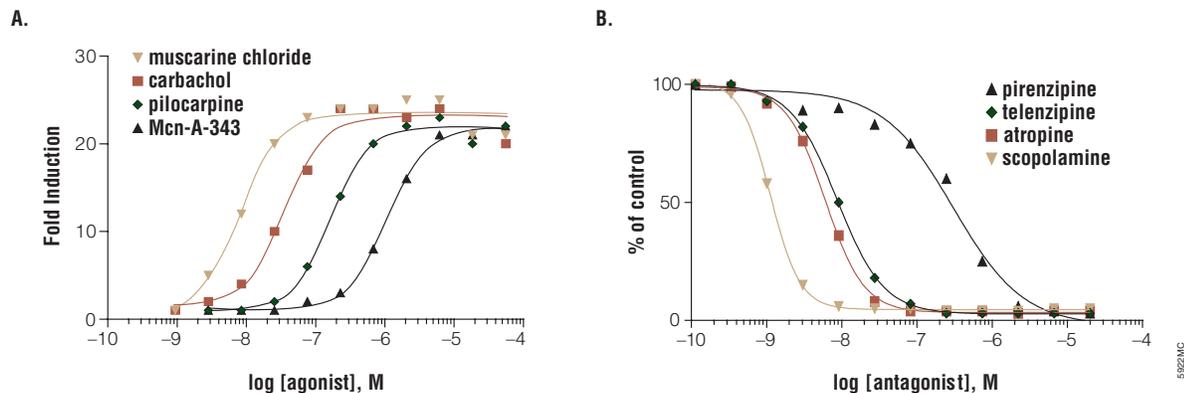


Figure 4. Ranking agonists and antagonists. The doubly transfected, stable HEK293 clones containing pGL4-RE-*luc2P* construct, where RE is NFAT, and pF9A-M3R-hR*luc*-Neo (NFAT-*luc2P*/M3R) were plated in 384-well plates at 10,000 cells/well. Modulators were serially diluted 1:3, added to wells in replicates of 8, incubated 6 hours, then assayed using Dual-Glo™ Assay System (Cat.# E2920). % control = 30nM muscarine chloride vs. muscarine chloride + antagonist. Fold induction = induced/uninduced Firefly RLU, normalized to *Renilla* RLU. Luciferase activity was measured on the Berthold® Mithras LB 96V Luminometer.

standard deviations above the average of the uninduced control. Data show that the screen readily identified hits targeted toward both the specific receptors and endogenous receptors of the host HEK293.

The screen was also performed for CRE-*luc2P*/DRD1 and CRE-*luc2P*/M3R. There are 73 hits common to both screens, suggesting that they are targeted to the common endogenous receptors. One hundred hits were identified specific for DRD1 and 30 hits for M3R. Importantly, all but two annotated agonists (dihydraxidine and SKF76560) for DRD1, and all annotated agonists for M3R, were identified.

Dose-response studies showed that dihydraxidine was a true agonist for DRD1 at low concentration, but apparent induction decreased after 2µM most likely due to toxicity, as shown by the corresponding decrease of the control reporter (*Renilla* luciferase; Figure 6). SKF76560 was listed as “an atypical agonist”, having antagonist activity in vitro and agonist activity in vivo. A titration study of this compound did not show induction up to 50µM (data not shown).

Improve Data Quality with Internal Control

The dual-reporter luciferase GPCR assay method has a built-in control using *Renilla* luciferase. Normalization of firefly luciferase activity with *Renilla* luciferase activity assists in compensating for potential variability in cell density and nonspecific effects caused by the compounds. However, since the nonspecific effects may not affect the expression of both reporter genes equally, a ratiometric presentation of the reporter data cannot reliably eliminate false hits.

We found that a more practical use of this internal control is to identify anomalous samples, which are revealed by a significant deviation of the *Renilla* luciferase activity from the norm of the sample set. For example, when we plotted both the firefly

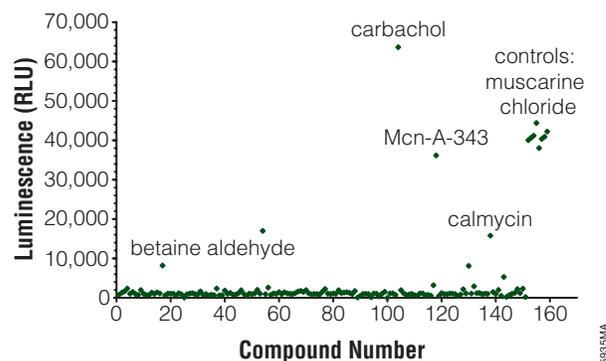


Figure 5. Screening yields receptor- and pathway-specific hits. A representative sample from the LOPAC library agonist screening in the NFAT/M3R doubly transfected, stable cell line is shown. The LOPAC chemical compound library was dispensed into four 384-well plates (n=1) at a final concentration of 10µM per compound using a TeMo™ multichannel pipettor. Each plate included agonist control (n=8) and uninduced controls (n=8). Cells were added at 10,000 cells/per well, and were harvested 8 hours post treatment. Firefly and *Renilla* luciferase activity were assayed with the Dual-Glo™ Assay System (Cat.# E2920), using a Tecan Safire2™ reader for both assays.

luciferase activity and *Renilla* luciferase activity for the sample plate shown in Figure 5, we found that four wells had significantly lower *Renilla* activities (Figure 7). Three out of the four wells also exhibited reduced firefly activities (outlined in yellow), indicating a common cause such as low cell density or compound toxicity. One of the four wells (outlined in green) gave moderate activation of firefly activity despite the reduced *Renilla* activity, suggesting that this compound, calcimycin, might be a stronger hit and should be tested further.

In fact, we followed up with a similar case in the CRE-*luc2P*/DRD1 screen. Dihydraxidine was not scored as a hit at 10µM based on firefly luciferase activity but had lower *Renilla* luciferase activity. A titration of dihydraxidine showed that it is

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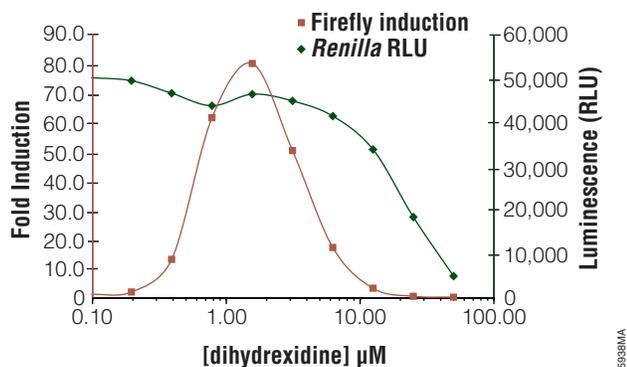


Figure 6. *Renilla* luciferase internal control indicates compound toxicity. The doubly transfected, stable HEK296 derived cell line containing CRE-*luc2P* and expressing DRD1 was plated in a 96-well plate at 10,000 cells/well. Dihydroxidine was diluted in a 1:2 series and added to wells (n=4 per concentration). Cells were harvested 4 hours post treatment and assayed for firefly and *Renilla* luciferase activity using the Dual-Glo™ Assay System (Cat.# E2920) and read on the GloMax™ 96 Microplate Luminometer (Cat.# E6501). Fold induction is calculated as the average firefly value of treated cells/ average firefly value of mock-treated cells at each concentration.

a strong agonist at concentrations up to 2μM (Figure 6). The level of induction decreased as concentration increased beyond 2μM. Similar decreases at these high dihydroxidine concentrations were observed for *Renilla* luciferase activity. Therefore, dihydroxidine appeared as a false negative in the assay and was identified as an agonist in a follow-up titration experiment.

Summary

We have shown that the dual-reporter method is well suited for studying modulators of the different GPCR signaling pathways in a high-throughput screening format. The assays are of high quality, as demonstrated by the robust induction and high Z'-factor values. GPCR modulators can be readily evaluated for their potency as agonists or antagonists using

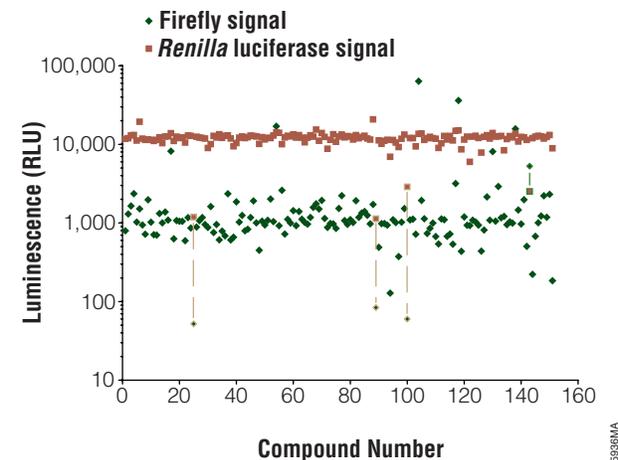


Figure 7. *Renilla* luciferase as an internal control. A representative sample of the LOPAC screening in the NFAT/M3R cell line shows instances where the *Renilla* RLU values for certain samples are outside the range of the average RLU. In the wells that are highlighted yellow, both firefly and *Renilla* RLU are low. Highlighted in green is a well corresponding to a strong agonist in the NFAT signaling pathway where the low *Renilla* values may explain the lower-than-expected firefly values. In these cases, the *Renilla* value is an indicator that the firefly results are suspect and should be evaluated further.

this assay system. In a mock HTS screen for agonists, the robust induction of reporter expression enables the detection of both full and partial agonists. The incorporation of *Renilla* luciferase as internal control reporter provides additional advantages over single reporter assays by allowing internal validation of screening data and identification of suspected samples for further analysis. ■

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Protocols

Dual-Glo™ Luciferase Assay System Technical Manual #TM058 (www.promega.com/tbs/tm058/tm058.html)

pGL4 Luciferase Reporter Vectors Technical Manual #TM259 (www.promega.com/tbs/tm259/tm259.html)

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