USING LUCIFERASE ASSAYS TO STUDY G-PROTEIN-COUPLED RECEPTOR PATHWAYS AND SCREEN FOR GPCR MODULATORS

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Introduction

Luciferase reporter assay systems are successfully used in the drug discovery process against G-protein-coupled receptors (GPCRs). These receptors form one of the most important drug target classes, with approximately 50% of currently marketed medicines targeted to GPCRs (1). Moreover, the functions of more than 150 orphan GPCRs in the human genome remain to be defined. Therefore, GPCRs continue to command tremendous interest in drug discovery. Luciferase reporters are useful for studying GPCR signaling pathways and screening for modulators of GPCR activity.

GPCRs interact with heterotrimeric G proteins at the plasma membrane, and upon stimulation by an agonist, lead to dissociation of G-protein subunits. Major G-protein families regulate different intracellular second messenger pathways based on the type of Gα subunit involved. These second messenger pathways in turn activate a range of effector systems to change cell behavior, including regulation of gene transcription. This affords the means for coupling luminescence to receptor activity (Figure 1, see references 2 and 3 for reviews).

Luciferase is the reporter of choice because of its sensitivity, dynamic ranges, fast and easy quantitation, and lack of endogenous activity.

Studying GPCR Pathways

Reporter-gene systems have been widely used for studying gene regulation in response to GPCRs. There are many reporter genes available, including alkaline phosphatase, chloramphenicol acetyltransferase, β-galactosidase, β-glucuronidase, β-lactamase, green fluorescent protein, luciferase and secreted alkaline phosphatase. Among them, luciferase is the reporter of choice because of its sensitivity, dynamic ranges, fast and easy quantitation, and lack of endogenous activity (3). Furthermore, the Dual-Luciferase® Reporter Assay System (Cat.# E1910) allows facile measurements of two luciferase activities in the same sample. In a typical experiment, firefly (*Photinus pyralis*) luciferase is used to monitor the transcription, whereas another luciferase from sea pansy (*Renilla reniformis*) is used as internal control of the transcriptional baseline and cell viability.

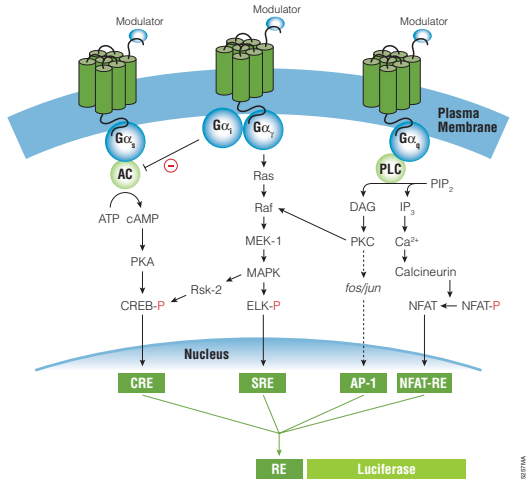

Figure 1. Schematic diagram showing GPCR signaling pathways. Upon stimulation, $G\alpha_s$-coupled receptors activate adenylyl cyclase (AC) resulting in an increase in cAMP; $G\alpha_i$-coupled receptors inhibit AC; the βγ subunits activate the MAP kinase pathways; $G\alpha_q$-coupled receptors activate phospholipase C (PLC) to produce IP3, 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-cells response element.

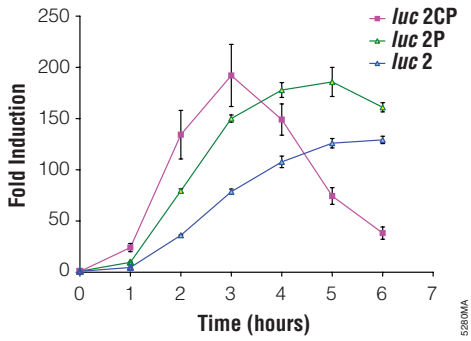

Figure 2. Destabilized luciferases increase the response and reduce the time to maximum induction. *luc2*, firefly luciferase; *luc*2P, firefly luciferase with hPEST sequence; *luc*CP, firefly luciferase with hCL1 and hPEST sequences. These luciferases were used to monitor the response of cAMP Response Element (CRE). A HEK293 cell line stably transfected with CRE-*luc* constructs was induced with 1µM isoproterenol/ 100µM Ro-20-1724, and samples were harvested every hour for quantifying luminescence.

Destabilized luciferase reporters improve the responsiveness to transcription dynamics. By fusing protein degradation signals hPEST and hCL to the C-terminus of luciferase, the

Luciferase-Based GPCR Assays for Drug Discovery

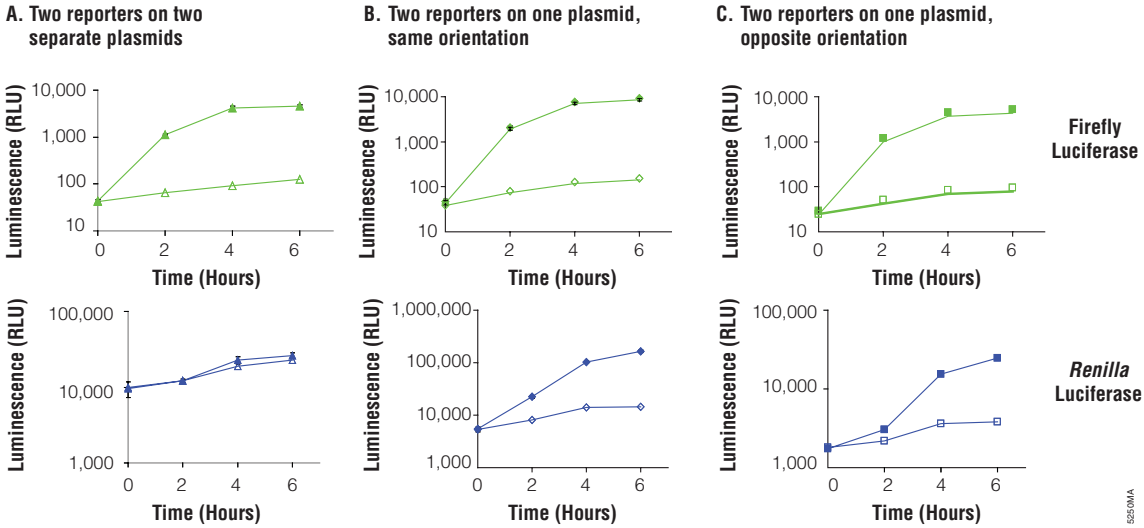

Figure 3. CRE-induction of firefly and *Renilla* luciferase activities using two separate plasmids (**Panel A**), one plasmid with both luciferase genes in the same (**Panel B**) and opposite orientations (**Panel C**). Open symbols = uninduced; Closed symbols = induced.

Figure 4. A diagram of two plasmids involved in the Dual Luciferase® GPCR assay. RE, response element/promoter; *luc*2P, Rapid Response™ firefly luciferase with PEST sequence; PSV40, SV40 promoter; PCMV, CMV promoter; Rluc-NEOr, *Renilla* luciferase and neomycin resistance gene fusion.

resulting Rapid Response™ luciferases give larger relative response in a shorter time than traditional luciferase reporters. For example, the destabilized reporters produce improved response kinetics for Gα_S-coupled receptors (Figure 2, reference 3). The action of these receptors, which modulate intracellular cAMP levels, is commonly coupled to luciferase transcription by a cAMP response element (CRE).

A high-throughput GPCR CRE assay typically employs a stable cell line with CRE/promoter-luciferase integrated into the chromosome. In most cases, expression of the GPCR of interest is also needed when there is low endogeneous expression. Special luciferase reagents can extend the glo-kinetics of the luciferase reaction to produce stable light output for hours. As more HTS assay technologies for GPCRs have emerged, the luciferase reporter gene system remains a preferred method (see reference 5 for review and recommendation).

Reduce False Positives

When screening for downregulated responses (e.g., antagonist response of GPCR), cytotoxicity of compounds can reduce net reporter expression, resulting in false positives. Since net expression of an internal control reporter would also be reduced, these false positives can be avoided using a Dual-Luciferase® Assay. Configuring both luciferases on a single vector is not recommended because of cross-interference. *Renilla* luciferase, serving as internal control under an SV40 promoter cross-responds to the CRE-induction when placed in the same vector as CRE-firefly luciferase. However, no such cross-induction occurs when the two luciferases are placed on two separate plasmids (Figure 3).

Therefore, we have developed a strategy to construct stable cell lines expressing two luciferases on two plasmids. One plasmid expresses the firefly luciferase gene 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 4).

Reduce Assay Noise

We demonstrated this Dual-Luciferase® method using dopamine receptor D1 (DRD1) as GPCR and HEK293 as the host cell line. pGL4-CRE-*luc*2P was constructed and used to transfect HEK293 cells. Hygromycin-resistant stable clones were generated, and one was chosen based on both expression levels and CRE-induction by isoproterenol. This stable CRE-*luc*2P cell line was then transfected with pR*luc*-Neor-DRD1. Hygromycin and neomycin double-resistant clones were isolated, and one clone was chosen based on induction by the DRD1 agonist SKF38393, yielding the final CRE-*luc*2P/DRD1 HEK293 cell line.

To evaluate the quality of a GPCR assay for HTS using this cell line, the Z´-factor was determined as follows:

$Z' = 1 - [(3*SDHigh + 3*SDLow)/ (AverageHigh - AverageLow)]$.

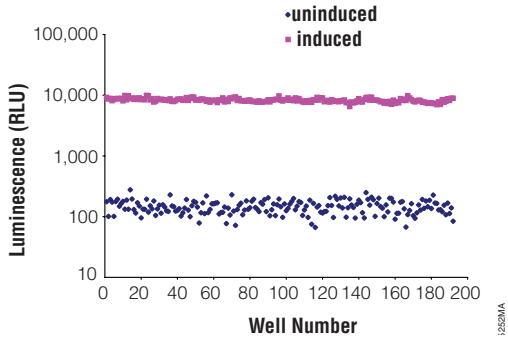

Figure 5. GPCR assay using CRE-*luc*2P/DRD1 HEK293 cell line in a 384-well format. Cells were seeded with 20µl of 10,000 cells/well. One half of the plate was used for the uninduced control, 100µM Ro-20-1724 only. The other half was induced with 1µM SKF38393 and 100µM Ro-20-1724. Cells were incubated for 4 hours. Luciferase activity was determined using the Dual-Glo™ Luciferase Assay System and the GENios-Pro luminometer. Firefly luciferase activity was normalized to *Renilla* luciferase activity using the following formula: Relative Light Units (RLU) = [sample firefly RLU/(sample R*luc* RLU/average R*luc* RLU)]. The Biomek® FX liquid handling system was used for compound and luciferase reagent additions.

The closer the Z´-factor is to 1, the better the assay quality. In general, values greater than 0.5 are acceptable for HTS (6). Our assay gave a Z´-factor of 0.77 in a 384-well format using firefly luciferase/*Renilla* luciferase ratiometric measurements (Figure 5). This was increased from a Z´-factor of 0.55 when firefly luciferase results were used without normalization, demonstrating improvement of assay quality using the two-luciferase strategy.

The CRE-*luc*2P/DRD1 HEK293 cell line showed dose-dependent response to both the agonist (SKF38393) and antagonist (SCH23390) of DRD1 (Figure 6). The EC_{50} of 2.5×10^{-7}M for SKF38393 and IC_{50} of 6.8×10^{-9} M for SCH23390 are consistent with previous reports (7).

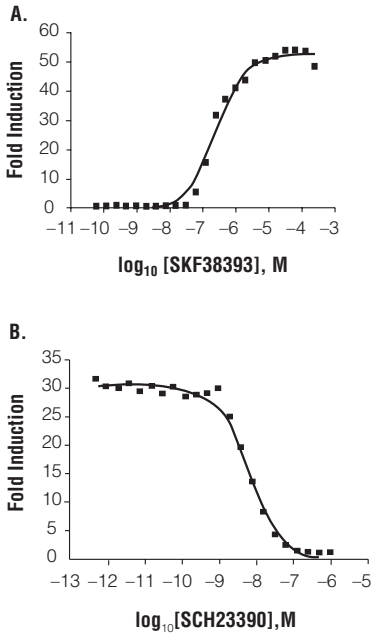

Figure 6. Dose-response curve of agonist and antagonist to DRD1 using CRC-*luc*P/DRD1 HEK293 cell line and Dual-Luciferase® Assay. Cells were plated at 10,000 cells/well in a 96-well plate. Serial dilutions of agonist or antagonist were added to replicates of four wells. Luminescence was measured after four hours with Dual-Glo™ Luciferase Reagent using a Veritas™ Luminometer. Fold induction was calculated as average induced RLU/average uninduced RLU.

Summary

Luciferase assay systems have been successfully used for studying GPCR signal pathways and high-throughput screening of GPCR modulators. We further improved performance by incorporating an internal control luciferase in a stable cell line to reduce false-positive hits and normalize well-to-well variations. Destabilized luciferase is more dynamic and used to reduce the assay time. The demonstrated Dual-Luciferase® cells enable rapid screening with improved data quality. ■

References

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Acknowledgments

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