

# MULTIPLEXING USING DUAL LUCIFERASE REPORTERS FOR GPCR ASSAYS

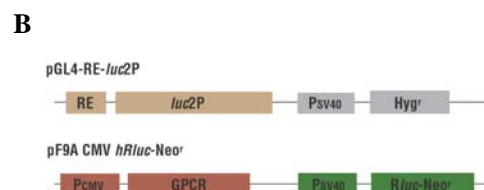
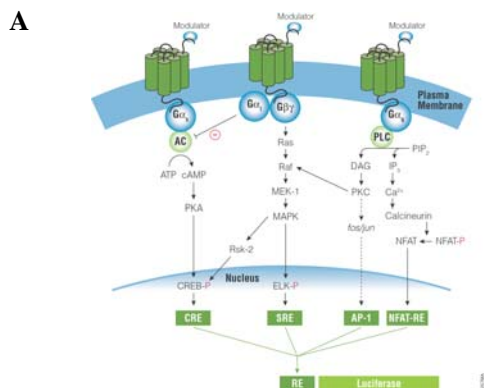
Frank Fan, Denise Garvin, Aileen Paguio, Brad Swanson, Tracy Worzella and Keith Wood  
 Promega Corporation, 2800 Woods Hollow Road, Madison WI 53711 Email: frank.fan@promega.com



## ABSTRACT

Bioluminescent reporter technologies are uniquely suited for high throughput screening due to their inherently high sensitivity, wide dynamic range and low susceptibility to compound interference. Improvement of data quality and reduction of false hits can be achieved by incorporating a control reporter (e.g. a second luciferase). Introducing both reporters into the cell on the same plasmid backbone can result in aberrant expression from cross interference between promoters and response elements. Therefore we have developed a strategy of generating dual luciferase stable cell lines for GPCR assays using a two plasmid system. Plasmid one features both a firefly luciferase gene regulated by the response element of interest (e.g. CRE or NFAT-RE) and a hygromycin selectable marker. The second plasmid expresses the target GPCR (e.g. muscarinic receptor) and a Renilla luciferase-neomycin selectable marker fusion. Destabilized luciferase reporters provide significant benefit by reducing assay time, which limits the duration of cytotoxic compound interactions. Multiplexing using this dual luciferase assay system enables rapid screening and improves data quality.

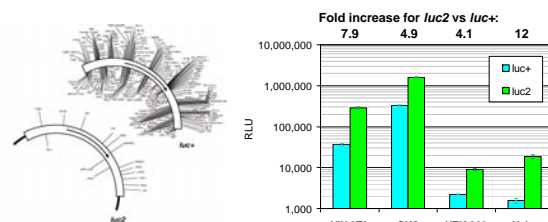
## Dual Luciferase GPCR Assay



Diagrams of GPCR signaling pathway (A) and the of two plasmids involved in the dual-luciferase GPCR assay (B).

RE, response element/promoter; luc2P, destabilized firefly luciferase with PEST sequence (Pro-Glu-Ser-Thr); P<sub>SV40</sub>, SV40 promoter; Hyg<sup>r</sup>, hygromycin resistance gene; P<sub>CMV</sub>, CMV promoter; Rluc-Neo<sup>r</sup>, Renilla luciferase and neomycin resistance marker fusion. PEST sequences are associated with rapidly degraded proteins.

## Enhanced Brightness and Efficiency of Reporter Gene



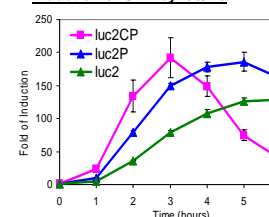
The coding sequence for the firefly luciferase was optimized for improved expression in mammalian cells. Consensus regulatory sequences such as transcription factor binding sites were also removed to reduce the risk of off-target expression.

## Rapid Response™ Luciferases Increases Reporter Dynamics

### Rapid Response™ reporter genes



### Induction of CRE by Iso/Ro



Versions of the luc2 gene containing protein degradation sequences were used to increase reporter dynamics (left). Stable HEK293 cells expressing CRE-luc2, CRE-luc2P or CRE-luc2CP were induced with 1 μM isoproterenol/ 100 μM Ro-20-1724. Samples were harvested every hour for quantifying luminescence.

## High Quality HTS Assays for GPCR's

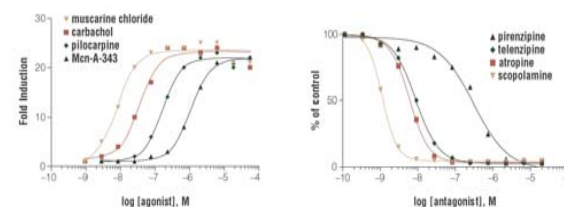
Response Element	Receptor	G-protein Subunit	Agonist		Antagonist	
			Fold Change	Z' in 384-well	Fold Change	Z' in 384-well
CRE	DRD1	Gαs	134	0.80	136	0.80
CRE	M3R	Gαs	107	0.82	30	0.64
CRE	DRD2	Gαi	78	0.80	83	0.80
NFAT	M1R	Gαq	23	0.67	28	0.72
NFAT	M3R	Gαq	20	0.73	14	0.63

The doubly transfected, stable HEK293 cells expressing RE-luc2P and various receptors were used. Agonist and antagonists are:

**For CRE-luc2P:** DRD1: dopamine, SCH23390  
 M3R: carbachol, 4-DAMP

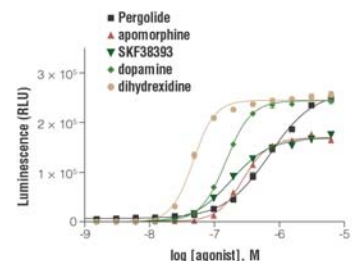
**For NFAT-RE-luc2P:** MIR: muscarinic chloride, scopolamine  
 M3R: muscarinic chloride, scopolamine

## Ranking Agonists and Antagonists



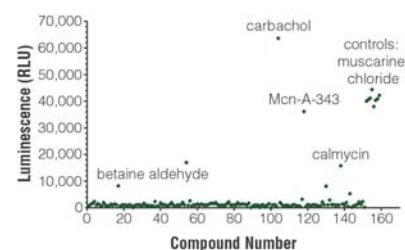
The double stable HEK293 cells expressing NFAT-RE-luc2P and muscarinic receptor 3 (M3R) were plated in 384-well plates at 10,000 cells/well. Modulators were serially diluted 1:3 and added to the well. Cells were harvested 6 hours post treatment and assayed using Dual-Glo™ Assay System. % control = 30nM muscarine chloride vs. muscarine chloride + antagonist. Fold induction = induced/uninduced Firefly RLU, normalized to Renilla RLU.

## Detecting Partial Agonists



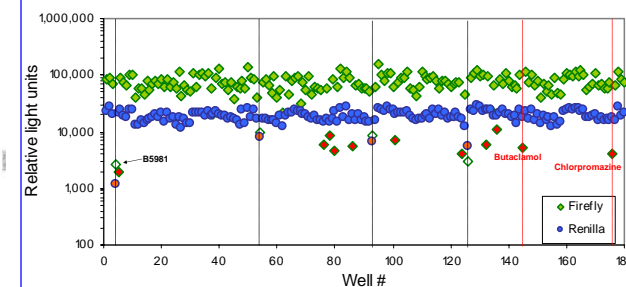
The double stable HEK293 cells expressing CRE-luc2P and dopamine receptor, D1 were plated into a 96 well plate. Each dopamine agonist was serially diluted 1:2 and added to the well. Cells were harvested 4 hrs post treatment and assayed with Dual-Glo™ Assay System. The results are consistent with the ranking of these agonists by other methods.

## Large Dynamic Range Allows Reliable Identification and Ranking of Assay Hits



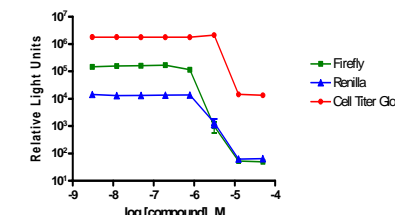
A representative sample from the LOPAC library agonist screening using HEK293 cells expressing NFAT-RE-luc2P and M3R. The LOPAC chemical compound library was dispensed into four 384-well plates at a final concentration of 10μM per compound. Cells were added at 10,000 cells/per well, and were harvested 8 hours post treatment. Firefly and Renilla luciferase activity were assayed with the Dual-Glo™ Assay System.

## Multiplexing with Internal Control Reporter Renilla Luciferase Improves Data Quality



The LOPAC library was screened for dopamine receptor, D1 (DRD1) antagonists using double stable HEK293 cells expressing CRE-luc2P and DRD1. The DRD1 agonist, dopamine, was added at a concentration of 5 μM followed by 10 μM of each LOPAC compound. Shown here are 15 hits based on firefly luciferase activities (◇, ◇); two are reported antagonists for DRD1 (Butaclamol and Chlorpromazine), four were found to also cause reduction in Renilla luciferase activities in the same well (◇, ◇), suggesting false positive. One of these, B5981, was confirmed to be cytotoxic (see below).

## Internal Control Reporter Renilla Luciferase Indicates Compound Toxicity



Compound B5981 showed inhibitory effects on firefly luciferase, Renilla luciferase and cellular ATP levels. B5981 was serially diluted 1:4 into 384 well plates beginning with 50 μM (n=4). HEK293 cells expressing CRE-luc2P and DRD1 were added at 10,000 cells/well. Plates were incubated for 4 hours, then assayed with Dual-Glo™ Luciferase Assay. A duplicate plate was assayed with CellTiter-Glo® Luminescent Cell Viability Assay.

## SUMMARY

- New generation of firefly luciferase gene (*luc2*) and its Rapid Response™ versions provided brighter luminescence and improved dynamics.
- Dual luciferase assays for receptors, using either NFAT or CRE pathway, exhibit good Z' values and dynamic range for both agonists and antagonists.
- Large dynamic range allows robust identification and ranking of GPCR modulators.
- Multiplexing with the Dual Luciferase System improves data quality by providing an internal control reporter to simultaneously monitor for cytotoxicity.