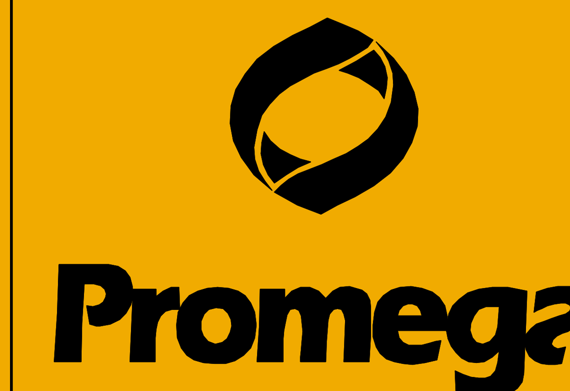
SRF-RE reporter assay for G_{12}-RhoA signaling pathway

Jey Cheng, Denise Garvin, Pete Stecha, Keith Wood, and Frank Fan

Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, USA

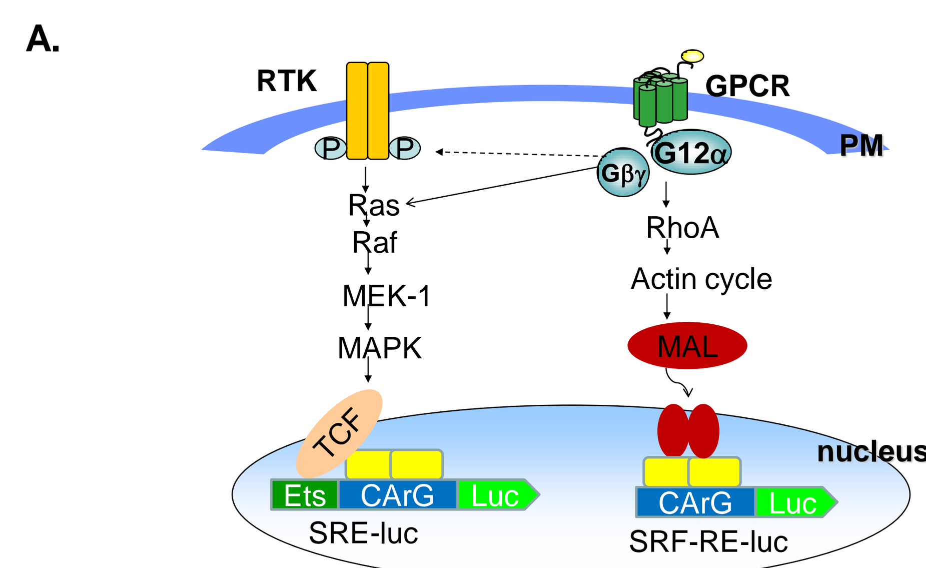


Abstract

The G_{12} subfamily of heterotrimeric G protein has been the subject of intense interest in both basic science and drug discovery research due to its involvement in regulating cell proliferation, cell migration and metastatic invasion, whereas not many drug-screening assays for G_{12}-linked pathway is available. Bioluminescent reporter assays are frequently used in high throughput screening due to their inherently high sensitivity, wide dynamic range and low susceptibility to compound interference. Here, We have incorporated the technical advancement of luciferase reporter genes and vectors to build an novel SRF-RE luciferase construct for RhoA signaling pathways. Large dynamic range of SRF-RE luciferase reporter assay allows efficient screening for GPCR modulators for G_{12} pathways. The dual luciferase GPCR assay system, whereas the second plasmid expresses the target GPCR and Renilla luciferase as internal control, improves data quality by providing simultaneous monitoring of nonspecific effects such as cytotoxicity.

Dual Luciferase SRF-RE Reporter Assay for RhoA Signaling

A.



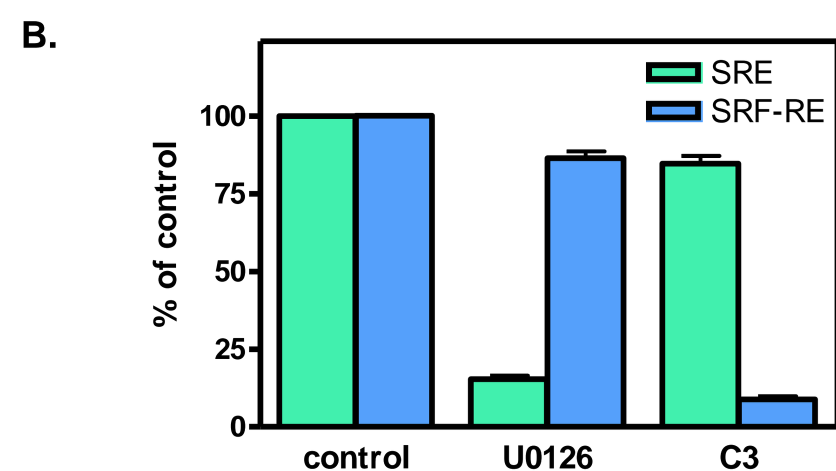
SRF-RE | *luc2P* | P_{SV40} | Hyg^r

SRF-RE-*luc2P* reporter responds to $G_{12\alpha}$-linked RhoA signaling pathway.

P_{CMV} | GPCR | P_{SV40} | *hRluc-Neo*^{r}

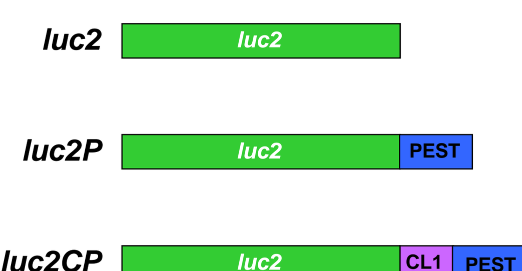
pF9A Vector expresses the GPCR of interest and Renilla-neo fusion control protein for normalization.

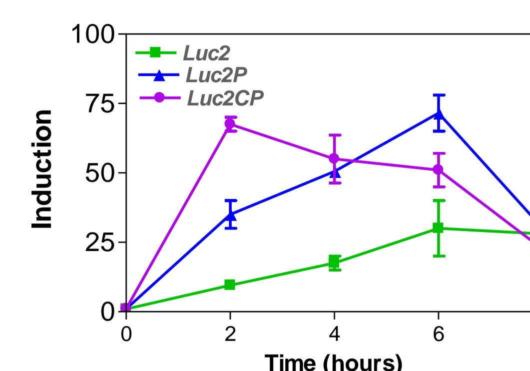
B.



HEK293 cells were transiently transfected with SRE-*luc2P* or SRF-RE-*luc2P* together with Renilla luciferase in 96-well plates. Cells were pretreated with/without U0126 (MEK inhibitor) or C3 Transferase (RhoA inhibitor), then induced with 10ng/ml PMA for SRE or 20%FBS for SRF reporter assay. Firefly luciferase activity was quantified six hours after induction using the Dual-Glo™ Assay System.

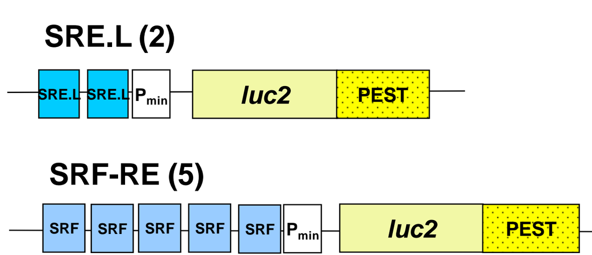
Rapid Response™ Luciferase Enhances Reporter Dynamics

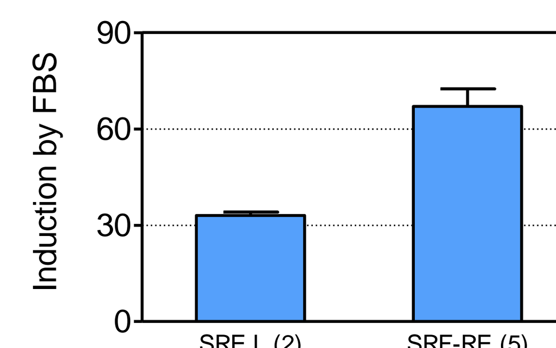




Versions of the *luc2* gene containing protein degradation sequences were used to enhance reporter dynamics. HEK293 cells transiently expressing SRF-RE-*luc2*, SRF-RE-*luc2P* or SRF-RE-*luc2CP* were induced with 20%FBS. Firefly luciferase activity was quantified every other hour for eight hours after induction using Luciferase Assay System.

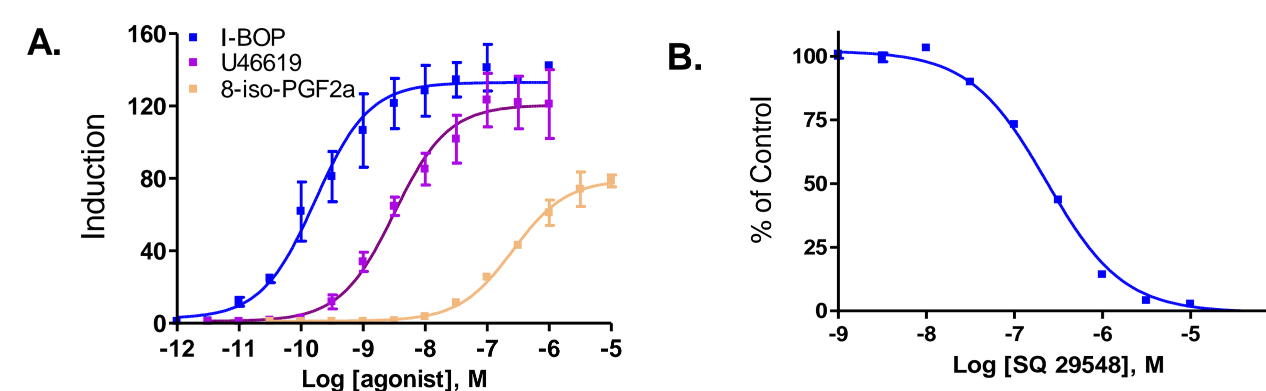
Optimized Reporter Sequences/Repeats Further Improves Reporter Dynamics





HEK293 cells were transiently transfected with SRE.L(2)-*luc2P* or SRF(5)-RE-*luc2P* together with Renilla luciferase in 96-well plates. Cells were induced with 20%FBS. Firefly luciferase activity was quantified six hours after induction using the Dual-Glo™ Assay System.

Agonist and Antagonist Assay



HEK293 cells were transiently transfected with SRF-RE-*luc2P*, thromboxane A_2 receptor and Renilla luciferase, and serum starved overnight. Twenty four hours after transfection, firefly luciferase activity was measured after six hours induction with addition of 1:3 serial dilutions of agonists (A), or pretreatment with antagonists before induction with 0.1 μM U46619 (B) using the Dual-Glo™ Assay System in 96-well format. For agonist assay, fold Induction = induced Firefly RLU/uninduced Firefly RLU, normalized to *Renilla* RLU. For antagonist assay, % of Control=[Firefly RLU (antagonist+agonist)/Firefly RLU (agonist alone)] ×100

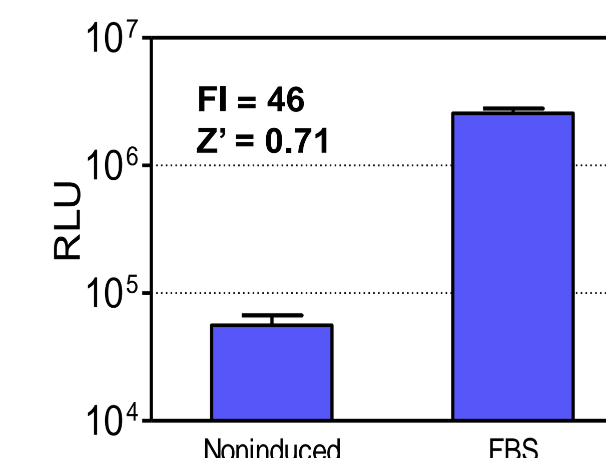
Summary of Fold change and EC_{50} of Various GPCR/Agonist Pairs

GPCR	G protein subunits	agonist	Fold Induction	EC_{50} (M)
LPA receptor	G_{12}, G_q	LPA	27	3.0×10^{-7}
Endothelin-B receptor	G_{12}, G_q	ET-1	11	8.0×10^{-10}
Thromboxane A_2 receptor	G_{12}, G_q	U46619	91	5.2×10^{-9}

HEK293 cells transiently expressing SRF-RE-*luc2P*, Renilla luciferase and various endogenous (LPA receptor) or exogenous receptors (endothelin-B receptor and thromboxane A_2 receptor) were induced with agonists for six hours and analyzed in 96-well format. Fold Induction = induced Firefly RLU/uninduced Firefly RLU, normalized to *Renilla* RLU.

High Quality HTS assay

Day 1	Plate cells in T75 flask in complete medium
Day 2	• Transfection in T75 flask • After 4 hrs, replate cells into 384 well plate, 20μl per well in serum-starving medium
Day 3	• Add 5μl compounds (5) per well • 6hr induction • Add 25μl One-Glo reagent, wait 3-5 min • read



HEK293 cells transiently expressing SRF-RE-*luc2P and* Renilla luciferase were replated in serum-starving medium in 384-well plates. 24 hours after transfection, cells were induced with 20%FBS for six hours. Firefly luciferase activity was quantified using the Dual-Glo™ Assay System. Fold Induction = induced Firefly RLU/uninduced Firefly RLU, normalized to *Renilla* RLU. Z' was calculated as $1-[(3SD_{induced}+3SD_{uninduced})/(Ave_{induced}-Ave_{uninduced})]$

Summary

Together the new SRF-RE luciferase reporter vector provide an excellent tool for G_{12} pathway in research and drug discovery programs:

- Optimized SRF-RE sequence by deactivating the Ets binding domain of SRE allows specific detection of signaling via RhoA pathway.
- Use of Rapid Response™ *luc2P* gene improves reporter dynamics and reduces assay time.
- Optimized SRF-RE repeats further improves the dynamic response.
- Large dynamic range allows robust identification and ranking of GPCR modulators through G_{12} pathways.
- Assay in HTS format shows good Z' value and dynamic range.