Advancing Pathway Analysis With Custom Luciferase Reporters

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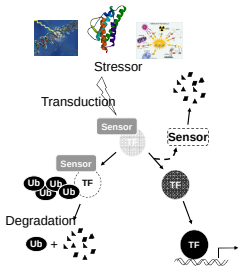
1. Abstract

To enable investigation of key cellular signaling pathways, Promega has developed a portfolio of bioluminescent reporter gene assays using Firefly and *Renilla* luciferases. In combination with best-in-class luciferase detection reagents, these genetic reporter systems enable interrogation of important cellular responses involved in cancer, inflammation, and CNS disease. To address specialized customer needs in our industrial and research markets, Promega has a new custom assay service team dedicated to applying these enabling technologies through strategic external research collaborations. The performance of this technology portfolio is presented, including novel applications of luciferase reporters to interrogation of cytokine, stress, and toxicity pathway responses.

2. Toxicity Pathways: A Common Pathway Architecture



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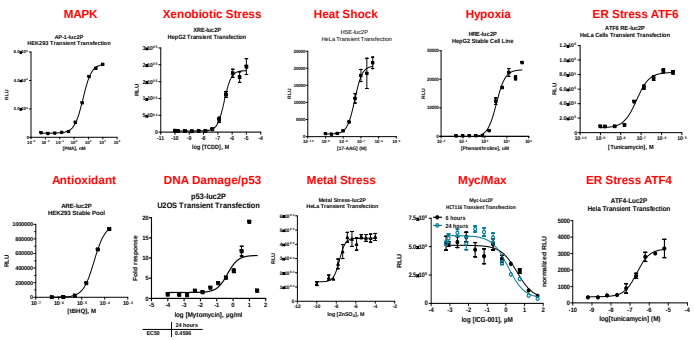
Latest Research Materials: Vectors and Cell Lines for Stress and Toxicity

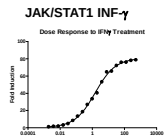
Toxicity Pathway	Response Element	Name
Antioxidant	ARE	pGL4.27[NRF2-luc2P/minP/Hygro] Vector
DNA damage/p53	p53	pGL4.27[p53-luc2P/minP/Hygro] Vector
ER stress	ATF4	pGL4.27[ATF4-luc2P/minP/Hygro] Vector
ER stress	ATF6	pGL4.27[ATF6-luc2P/minP/Hygro] Vector
ER stress	Xbp1	pGL4.27[Xbp1-luc2P/minP/Hygro] Vector
Heavy metal stress	MRE	pGL4.27[MTF1-luc2P/minP/Hygro] Vector
Heat shock	HSE	pGL4.27[HSE-luc2P/minP/Hygro] Vector
Hypoxia	HIF1	pGL4.27[HIF1-luc2P/minP/Hygro] Vector
MAPK	AP1	pGL4.27[AP1-luc2P/minP/Hygro] Vector
Xenobiotic stress	AhR	pGL4.27[XRE-luc2P/minP/Hygro] Vector
Myc	Myc/Max	pGL4.27[Mycluc2P/minP/Hygro] Vector

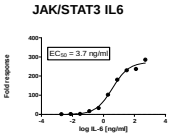
Toxicity Pathway	Response Element	Cell Line Background
Antioxidant	ARE	HEK293
Hypoxia	HIF1	HEK293
Hypoxia	HIF1	HepG2
MAPK	AP1	HEK293
*Ras/MEK-1	SRE	HEK293
*RhoA (Gα12/13)	SRF	HEK293

3. Superior Functional Performance in Transient Transfection



4. Cytokine and Pathway Analysis for Biologics





Latest Research Materials: Vectors and Cell Lines for Cytokines and Pathways

Cytokine Pathway	Response Element	Name
Hedgehog	GLI	pGL4[luc2P/Gli-RE/Hygro] Vector
JAK/STAT1/2 INF-α	ISRE	pGL4[luc2P/ISRE/Hygro] Vector
JAK/STAT1 INF-γ	GAS	pGL4[luc2P/GAS-RE/Hygro] Vector
JAK/STAT3 IL-6	SIE	pGL4[luc2P/SIE-RE/Hygro] Vector
PI3K/AKT	DBE	pGL4[luc2P/DBE-RE/Hygro] Vector
Multiple	C/EBP	pGL4[luc2P/C/EBP-RE/Hygro] Vector
Wnt	LEF/TCF	pGL4[luc2P/LEF/TCF-RE/Hygro] Vector
TGF-beta	SMAD3/4	pGL4[luc2P/SMAD3/4-RE/Hygro] Vector

Activator/pathway	Reporter Construct	Cell Background/marker
INF-γ:STAT1:STAT1	GAS-luc2P	HEK293/Hygro
INF-γ:STAT1:STAT1	GAS-luc2P	HepG2/Hygro
IL-6/STAT3:STAT3	SIE-luc2P	HEK293/Hygro
IL-6/STAT3:STAT3	SIE-luc2P	HepG2/Hygro

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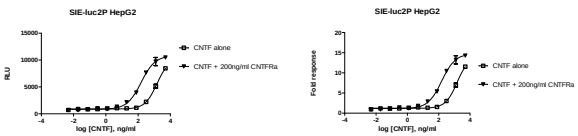
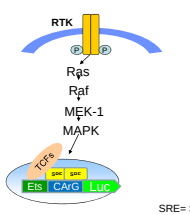
5. CASE STUDY #1: Detecting CNTFRα Using SIE-Luc2P/HepG2 Stable Cell Line

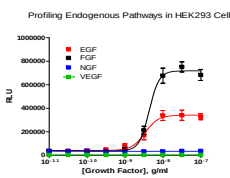


	CNTF alone	CNTF + 200ng/mL CNTFRa
Ec50, ng/mL	1496	154.2

Materials and Methods: Ciliary neurotrophic factor (CNTF) is a 23 kDa neurocytokine which is expressed in both the peripheral and central nervous system and provide hopes for its possible clinical application in the treatment of human neurodegenerative diseases. Like other cytokines, CNTF exerts its biological effects through the activation of a multichain receptor complex consisting of a ligand-specific subunit (CNTFRα), gp130 and the LIF receptor-β (LIFR-β). GloResponse™ SIE-Luc2P reporter is stably expressed in HepG2 cells where LIFR is endogenously expressed with no CNTFR detection. Stable cells were plated in assay media (MEM + 10% FBS) at 40K cells/80 ul/well in 96 well plate and incubated overnight at 37°C with 5% CO2. Next day,10ul of 10X soluble CNTFRα was added to appropriate wells (or media to untreated wells) with subsequent 10ul of 10X CNTF dose addition. Cells were incubated 6 hours prior to One-Glo substrate (Promega, Cat.# E6110) addition. As shown in the data, in the present of exogenously added soluble CNTFRα, LIF receptor bearing HepG2 cells respond to CNTF at lower doses, consistent to the published data.

6. CASE STUDY #2: Leveraging SRE-Luc2P/HEK293 Reporter Cell Line to Bioassay Development





SRE= Serum Response Element-luc2P (destabilized Fluc)

Materials and Methods: GloResponse™ SRE-Luc2P HEK293 cells were plated in 96 well plate overnight. Cells were stimulated with serially-diluted agonist and allowed to incubate approximately 18 hours. For detection of luciferase activity after stimulation, to 100uL of culture medium, an equal volume of ONE-Glo™ detection reagent was added to each well. After 10 minutes of equilibration, luminescence was measured on a GloMax™ Multi plate reader set to 0.5s of luminescence integration time.

7. Protein Stability Regulates Key Cytokine and Stress Response Pathways

Target Protein	$T_{1/2}$ Normal (min)	$T_{1/2}$ Induced (min)	Inducer
HIF1α	50	200-250	Hypoxia/mimetics
IκBα	100	5	TNFa/other inflammatory cytokines
p53	20	300-400	Genotoxic stress (UV/chemical DNA damage, etc)
Nrf2	10-15	30-40	Oxidative Stress
β-Catenin	<60	>200	Wnt
FOXO	>300	<60	Growth Factors
PDCD4	300	<60	Insulin / PI3K
c-Jun	<60	>200	Stress
c-Myc	20	300-400	Stress
c/EBP	<60	>300	LiCl

8. Protein Stability Reporters Using *Renilla* Luciferase Enable Signaling Pathway Measurements in Real-time

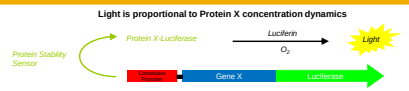


Figure 1

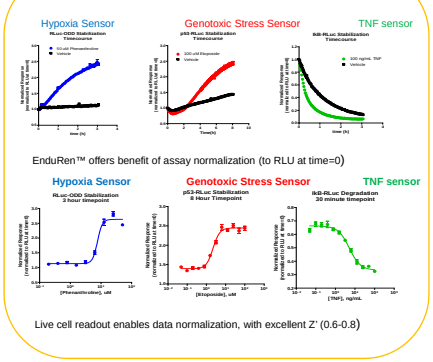
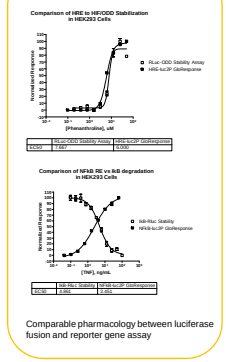


Figure 2



Materials and Methods: Measurement of inducible stabilization of various *Renilla* luciferase fusion proteins. **Figure 1**: HEK293 cells were transfected with pF5A DNA encoding humanized *Renilla* luciferase (Rluc) fusion proteins using Fugene HD and seeded in 96-well format. **Left panels**; cells were transfected with DNA encoding the ODD domain of HIF1a fused to the c-terminus of Rluc. To achieve appropriate expression levels of Rluc-ODD, reporter DNA was diluted into pGem3Z DNA to yield 50pg/well. **Middle panels**; cells were transfected with DNA encoding p53 fused to the N-terminus of Rluc at 0.5ng/well as described above. **Right panels**; cells were transfected with DNA encoding IkB fused to the N-terminus of Rluc at 50pg/well as described above. 24 hours post-transfection, cell medium was replaced with CO2-independent medium supplemented with 20uM EnduRen™ Reagent, followed by equilibration for 4 hours at 37C. Cells were then stimulated with the serially diluted agonist indicated, for the time indicated. Luminescence was then measured in real-time on a GLOMAX Multi+ Plate reader set to 5 minute measurement intervals. For data normalization, RLUs of each sample were normalized to the RLU values immediately after stimulation for that sample. **Figure 2**: Comparable pharmacology between luciferase fusion and reporter gene assay for measuring hypoxia response (top) and TNF response (bottom)..

9. Conclusions

- **Promega now offers fully validated Firefly luciferase reporter vectors for key toxicity and cytokine signaling pathways**
- **Modular solutions exist for the investigation of endogenous and exogenous receptor biology using GloResponse™ stable cell lines**
- **Novel pathway analysis tools are available using luciferase fusion technologies**
- **Promega's Custom Assay Service (CAS) team provides expertise in generation of custom vectors or stable cell lines encoding bioluminescent reporters**

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