TECHNICAL BULLETIN

GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line

Instructions for Use of Product
E8510

Revised 6/18
TB363

GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line

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1. Description

Luciferase reporter assays are used widely to investigate cellular signaling pathways and as high-throughput screening tools for drug discovery (1–3). The GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line[a–e] is a clonal derivative of Human Embryonic Kidney 293 (HEK293)[d] cells. These cells contain a luciferase gene (*luc2P*) under the control of a minimal TATA promoter with multiple Nuclear Factor of Activated T-cell response elements (NFAT-REs). NFAT-REs are the DNA-binding sequences for the NFAT family of transcription factors, which are responsible for regulating a variety of biological functions including cell proliferation, cytokine production, and cardiovascular development (4,5).

The GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line is designed for rapid and convenient analysis of any cellular response that results in modulation of NFAT activities. A typical example involves G-protein-coupled receptors (GPCRs) where modulators can lead to changes in intracellular Ca^{2+} levels, which in turn modulate the activities of NFAT and NFAT-RE-*luc2P* (see Section 6.A for details).

The GloResponse™-NFAT-RE-*luc2P* HEK293 Cell Line was generated by clonal selection of HEK293 cells stably transfected with the pGL4.30 [*luc2P*/NFAT-RE/Hygro] Vector (Cat.# E8481). This cell line incorporates improvements developed for the pGL4 family of reporter vectors for enhanced performance (6). The destabilized *luc2P* luciferase reporter is used to improve responsiveness to transcription dynamics. The *luc2P* gene is codon-optimized for enhanced expression in mammalian cells, and the pGL4 plasmid backbone has been engineered to reduce background reporter expression.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line	**2 vials**	**E8510**

Includes: Two vials of 2×10^6 cells in Freezing Media.

Cells are shipped frozen. If cells are not frozen upon arrival, contact Promega customer service immediately.

Place frozen cells in storage at or below −140°C (mechanical deep freeze or vapor phase liquid nitrogen) until you are ready to thaw and propagate them.

WARNING: Do not use cryotubes in the liquid phase of liquid nitrogen. Improper use may trap liquefied nitrogen inside the vial and lead to pressure buildup, resulting in possible explosion or biohazard release. Use appropriate safety procedures when handling and disposing of the cryotubes.

Product Warranty

Promega warrants that cells will be viable upon shipment from Promega for a period of thirty days provided they have been properly stored and handled during this period.

Handling Cells Upon Arrival

We strongly recommend that you propagate the cells using the provided procedure as soon as possible after receipt (Section 4). This will ensure the best cell viability and assay performance. The second vial is supplied as an immediate backup and is not intended for long-term storage.

Cell Line Stability

Cells may undergo genotypic changes resulting in reduced responsiveness over time in normal cell culture conditions. Genetic instability is a biological phenomenon that occurs in all stably transfected cells. Therefore, it is critical to prepare an adequate number of frozen stocks at early passages.

3. Before You Begin

3.A. Materials to Be Supplied by the User

Recommended Reagents

- DMEM, high glucose (Invitrogen Cat.# 11965-118)
- characterized fetal bovine serum (FBS; Hyclone Cat.# SH30071)
- DMSO (Sigma-Aldrich Cat.# D2438)
- hygromycin B (Invitrogen Cat.# 10687-010)
- phosphate-buffered saline (PBS; Invitrogen Cat.# 20012-050)
- TrypLE™ Express trypsin (Invitrogen Cat.# 12605010)
- Bright-Glo™ Luciferase Assay System (Promega Cat.# E2610) or Dual-Glo® Luciferase Assay System (Promega Cat.# E2920)
- PMA (Sigma-Aldrich Cat.# P8139)
- ionomycin (Sigma-Aldrich Cat.# I0634)

Supplies and Equipment

- tissue culture-treated, solid white, 96-well assay plate (Costar® Cat.# 3917)
- 15ml conical tubes
- cryovials
- tissue culture flasks
- class II biological safety cabinet
- hemacytometer
- humidified 37°C, 5% CO_2 incubator
- inverted microscope
- luminometer

3.B. Composition of Required Media and Buffers

Growth Medium

90% DMEM
10% FBS

Growth Medium + hygromycin B

90% DMEM
10% FBS
50µg/ml hygromycin B

Freezing Medium

80% DMEM
15% FBS
5% DMSO

Assay Medium

99% DMEM
1% FBS

PMA

1mg/ml in DMSO

ionomycin solution

1mM (0.75mg/ml) in DMSO

5X PMA/ionomycin solution

50ng/ml PMA + 5µM ionomycin in Assay Medium

5X DMSO

0.5% DMSO in Assay Medium

4. Maintenance of GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line

4.A. Thawing and Initial Culture Procedure

1. Rapidly thaw the cells by placing them at 37°C in a water bath with gentle agitation for 1–2 minutes.
   Note: Freezing Medium may be yellow immediately after thawing. This does not affect cell viability if these instructions are followed.
2. Decontaminate the vial by wiping it with 70% ethanol before opening in a class II biological safety cabinet.
3. Slowly transfer the vial contents into 10ml of Growth Medium (Section 3.B) in a sterile 15ml conical tube.
4. Centrifuge the cells at 500 × *g* for 5 minutes at 18°C.
5. Aspirate supernatant and resuspend the cell pellet in 12ml of 37°C prewarmed Growth Medium.
6. Transfer resuspended cells to a T75 flask.
7. At first passage, switch to Growth Medium + hygromycin B (Section 3.B).

4.B. Propagation of Cells

Cells should be maintained between 10% and 90% confluency in a 37°C, 5% CO_2 tissue culture incubator. This typically will require passaging the culture twice a week. The approximate cell number for 100% confluency for this cell line in a T75 flask is 1×10^7 cells. Media formulations are provided in Section 3.B. Volumes listed are for propagation in a T75 flask.

1. When cells have reached the appropriate density, aspirate the medium from the flask.
2. Wash once with 12ml 1X PBS.
3. Add 2ml of TrypLE™ Express trypsin. Evenly coat the flask surface containing the cells. Trypsinize for 2 minutes.
4. Using a microscope, verify that the cells have detached and clumps have completely dispersed.
5. Stop trypsinization by adding 10ml of Growth Medium.
6. Transfer cell suspension to conical tube. Determine cell number using a hemacytometer.
7. Pellet cells at 500 × *g* for 5 minutes at 18°C.
8. Aspirate supernatant and resuspend cells in Growth Medium + hygromycin B.
9. Seed new flasks at appropriate cell density, depending on the size of flask. For example, use 1×10^6 cells for a T75 flask.
10. Place flasks in 5% CO_2, 37°C incubator.

4.C. Freezing Cells

1. Grow cells to a density of 50% confluency. Replace Growth Medium + hygromycin B with Growth Medium (no hygromycin B) the day before harvest.
2. Harvest cells as described in Section 4.B. After the cells have detached, briefly centrifuge cells and resuspend them in Freezing Medium (Section 3.B).
3. Dispense 1.0ml per cryogenic vial.
4. Place vials in an insulated container (i.e., Styrofoam® or Nalgene® Mr. Frosty, Cat.# 5100-0001) for slow cooling, and store overnight at –80°C.
5. Transfer to liquid nitrogen tank or –140°C.

5. Performance Assay

This section outlines the recommended procedure to measure the performance of the NFAT-RE-*luc2P* HEK293 cell line in response to PMA/ionomycin in 384- or 96-well assay formats. Volumes should be scaled appropriately for different plates and the desired number of replicate samples for each condition. Dispense solutions with a multichannel pipettor whenever possible.

5.A. Cell Culture Preparation

Two or three days before performing the assay, split cells in growth medium so that they will be 100% confluent the day before the assay. The PMA/ionomycin control induction used in the Performance Assay works optimally when the cells are serum-starved. Do not change the medium after plating the flask.

5.B. Recommended Control and Treatment Conditions

Treatment or Control	Composition (per well in a 384-well plate)	Purpose
Unstimulated Control	Cells in 20µl Assay Medium (Section 3.B) and 5µl DMSO solution (Section 3.B)	Measures uninduced level of NFAT-RE reporter activity.
PMA/ionomycin-Stimulated	Cells in 20µl Assay Medium (Section 3.B) and 5µl 5X PMA/ ionomycin solution (Section 3.B)	Measures induced level of NFAT-RE reporter activity.
Cell-Free Control	25µl Assay Medium (Section 3.B)	Determines background luminescence of instrument.

5.C. Preparation of Induction and Control Solutions

1. Prepare 5X PMA/ionomycin solution in Assay Medium (50ng/ml PMA + 5µM ionomycin.) The final concentration in the well will be 10ng/ml PMA and 1µM ionomycin.
2. Prepare a 5X DMSO solution in Assay Medium (0.5% DMSO). The final concentration in the well will be 0.1% DMSO.

5.D. Performance Assay Protocol

(Protocol is for 384-well format; volumes for 96-well format are provided in parentheses.)

1. For a 384-well plate, add 5µl of the 5X DMSO solution to the Unstimulated Control wells (20µl for a 96-well plate).
2. Add 5µl of 5X PMA/ionomycin solution to PMA/ionomycin-Stimulated wells (20µl for a 96-well plate).
3. Add 25µl of Assay Medium to Cell-Free Control wells (100µl for a 96-well plate). Return the plate to the tissue culture incubator while preparing cells in Step 4.
4. Harvest as described in Section 4.B, Steps 1–8, using Assay Medium instead of Growth Medium. Resuspend cells in Assay Medium to a density of 5.0×10^5 cells/ml.
5. Add 20µl/well of cell suspension to Unstimulated Control wells and PMA/ionomycin-Stimulated wells (80µl for a 96-well plate).
6. Incubate the assay plate in a humidified 37°C, 5% CO_2 incubator for 18 hours.
7. Reconstitute reagents as directed in the *Bright-Glo™ Luciferase Assay System Technical Manual* (#TM052) to measure firefly luciferase activity.

   Note: If a doubly transfected, stable cell line expressing a target receptor of interest has been generated using the pF9A CMV *hRluc*-neo Flexi® Vector (Cat.# C9361) and both firefly luciferase and *Renilla* luciferase are to be read from the same sample, then use the Dual-Glo® Luciferase Assay System Reagent to measure luciferase activity.
8. Add 25µl of Bright-Glo™ Luciferase Assay Reagent to each well (100µl for a 96-well plate).
9. Incubate at room temperature for 10 minutes.
10. Measure luminescence using a luminometer, read for 0.5 second/well.

5.E. Data Analysis

The formula to calculate reporter gene induction by PMA + ionomycin is provided below.

$$\text{induction} = \frac{(\text{average Stimulated wells} - \text{average Cell-Free Control wells})}{(\text{average Unstimulated Control wells} - \text{average Cell-Free Control wells})}$$

5.F. Sample Data

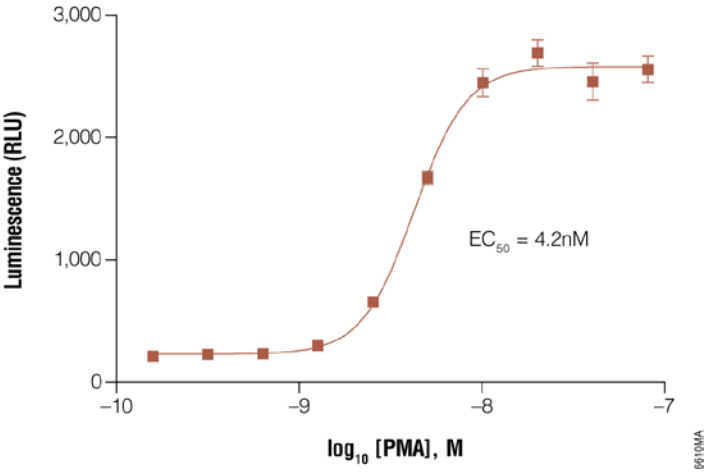

Figure 1. GloResponse™ NFAT-RE-*luc2P* HEK293 cells response to PMA titration. A total of 10,000 GloResponse™ NFAT-RE-*luc2P* HEK293 cells per well were dispensed into each well of a 384-well plate, and threefold serial dilutions of PMA were added to induce reporter gene expression. After 16 hours of induction in a tissue culture incubator, luciferase activity was quantified using the Dual-Glo® Luciferase Assay System Reagent on the Berthold® LB 96 V Luminometer. n = 8 for each data point.

6. Appendix

6.A. Using the GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line for GPCR Assays

GPCRs regulate a wide range of biological functions and form one of the most important target classes for drug discovery (7). GPCR signaling pathways can be categorized into three classes based on the G protein α-subunit involved: $G\alpha_s$, $G\alpha_{i/o}$ and $G\alpha_q$. The GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line can be used to study and configure screening assays for $G\alpha_q$-coupled GPCRs. For $G\alpha_s$- and $G\alpha_{i/o}$-coupled GPCRs, the GloResponse™ CRE-*luc2P* HEK293 Cell Line (Cat.# E8500) should be used.

Unmodified GloResponse™ Cell Lines can be used directly to measure activity of endogenous GPCRs present in the host HEK293 cells. For exogenous GPCRs, the gene for the receptor needs to be transfected into the cells and expressed. We recommend using the mammalian expression vector pF9A CMV *hRluc*-neo Flexi® Vector (Cat.# C9361, Figure 2) containing the receptor sequences to introduce the gene into the cells. This vector contains a fusion protein of *Renilla* luciferase (*hRluc*) and neomycin-resistance marker (neo), which can be used both for selection and as an internal reporter control during drug screening (8,9).

Examples of GPCR assays using this strategy are shown in Figure 3 and Table 1. The GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line was stably transfected with a muscarinic M3 receptor and tested on panels of agonists and antagonists (Figure 3). The results demonstrate the biologically relevant potency ranking.

6.A. Using the GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line for GPCR Assays (continued)

The GPCR assays configured using the GloResponse™ Cell Lines are amenable to high-throughput screening. These assays typically have greater response dynamics (fold of induction) than other assay formats and high Z´-factor values (Table 1; 10).

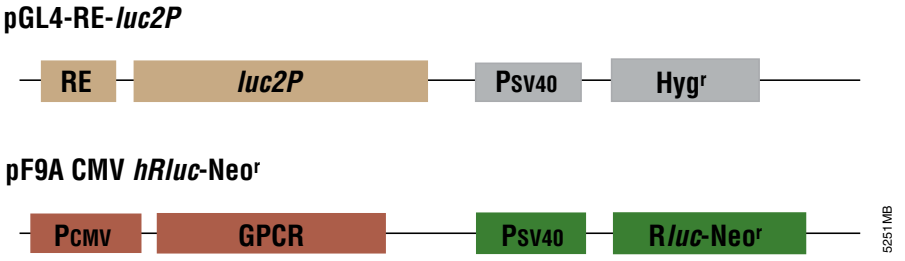

Figure 2. A diagram of two plasmids involved in the dual-luciferase GPCR assay. RE, response element/promoter; *luc2P*, destabilized firefly luciferase with PEST sequence (proline, glutamate, serine, threonine); P_{SV40}, SV40 promoter; Hygr, hygromycin-resistance gene; P_{CMV}, CMV promoter; *Rluc*-neor, *Renilla* luciferase and neomycin-resistance gene fusion. PEST sequences are associated with rapidly degraded proteins.

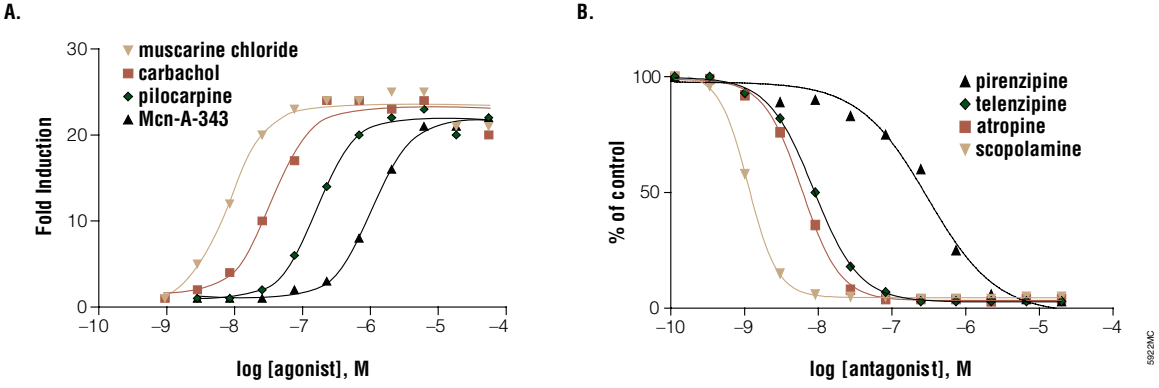

Figure 3. Ranking agonists and antagonists. A GloResponse™ NFAT-RE-*luc2P* clone stably expressing the muscarinic receptor M3 was plated in 384-well plates at 10,000 cells/well. Modulators (**Panel A**. Agonists; **Panel B**. Antagonists) were serially diluted 1:3, added to wells in replicates of eight, incubated for 6 hours, then assayed using the Dual-Glo® Luciferase Assay System (Cat.# E2920).
% of control = 30nM muscarine chloride v. muscarine chloride only + antagonist.
Fold induction = induced/uninduced firefly luminescence, normalized to *Renilla* luminescence. Luciferase activity was measured on the Berthold® Mithras LB 96V Luminometer.

Table 1. High-Quality Data from GloResponse™ NFAT-RE-*luc2P* HEK293 Cells.

Response Element	Receptor	G-Protein Subunit	Fold Induction	Z´-Factor Value in 384-Well Plates	Agonist
NFAT	M1R	$G\alpha_q$	23	0.67	muscarine chloride
NFAT	M3R	$G\alpha_q$	20	0.73	muscarine chloride

GloResponse™ NFAT-RE-*luc2P* cells were stably transfected with the pF9A CMV *hRluc*-neo Flexi® Vector expressing either the M1 or M3 muscarinic receptor. A high-performing clone for each was selected for further analysis based on induction by a 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 6 hours. Luciferase activity was determined using the Dual-Glo® Luciferase Assay System (Cat.# E2920) and quantified using a Berthold® LB 96 V luminometer. Induction was calculated as the average firefly stimulated luminescence/average mock-stimulated luminescence.

6.B. References

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2. Zhuang, F. and Liu, Y.H. (2006) Usefulness of the luciferase reporter system to test the efficacy of siRNA. *Methods Mol. Biol.* **342**, 181–7.
3. Hill, S.J., Baker, J.G. and Rees, S. (2001) Reporter-gene systems for the study of G-protein-coupled receptors. *Curr. Opin. Pharmacol.* **1**, 526–32.
4. Shaw, J.P. *et al.* (1988) Identification of a putative regulator of early T cell activation genes. *Science* **241**, 202–5.
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6. Paguio, A. *et al.* (2005) pGL4 Vectors: A new generation of luciferase reporter vectors. *Promega Notes* **89**, 7–10.
7. Klabunde, T. and Hessler, G. (2002) Drug design strategies for targeting G-protein-coupled receptors. *Chembiochem.* **3**, 928–44.
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10. Zhang, J-H., Chung, T.D.Y. and Oldenburg, K.R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4**, 67–73.

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6.C. Related Products

Product	Size	Cat.#
GloResponse™ CRE-*luc2P* HEK293 Cell Line	2 × 10^6 cells	E8500
GloResponse™ NF-κB-RE-*luc2P* HEK293 Cell Line	2 × 10^6 cells	E8520
Dual-Glo® Luciferase Assay System	10ml*	E2920
Bright-Glo™ Luciferase Assay System	10ml*	E2610
Steady-Glo® Luciferase Assay System	10ml*	E2510
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500
pF9A CMV *hRluc*-neo Flexi® Vector	20µg	C9361
cAMP-Glo™ Assay	300 assays*	V1501
PDE-Glo™ Phosphodiesterase Assay	1,000 assays*	V1361
Kinase-Glo® Luminescent Kinase Assay	10ml*	V6711
Kinase-Glo® Plus Luminescent Kinase Assay	10ml*	V3771

*Additional Sizes Available.

7. Summary of Change

The following changes were made to the 6/18 revision of this document:

1. Legal disclaimer information was updated.

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[b]U.S. Pat. No. 8,008,006 and European Pat. No. 1341808.

[c]Patent Pending.

[d]HEK293 cells were obtained under license from AdVec Inc.

[e]**Use of Genetically Modified Microorganisms (GMM)**

Information for European Customers: These products are genetically modified as described in Promega technical literature. As a condition of sale, use of this product must be in accordance with all applicable local guidelines on the contained use of genetically modified microorganisms, including the Directive 2009/41/EC of the European Parliament and of the Council.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.