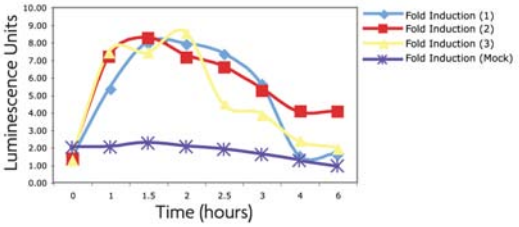
RapidReporter® pRR-High-CRE

Catalog No: 33007, 33008

Background:
The cyclic AMP response element binding (CREB) protein specifically recognizes the cAMP-responsive element (CRE) promoter site. CREB proteins activate transcription of target genes in response to a diverse array of stimuli, such as hormones, growth factors and neuronal activity1. Activation of CREB is mediated by a variety of protein kinases including protein kinase A (PKA) and mitogen-activated protein kinases (MAPKs) that phosphorylate CREB at the Serine 133 residue. Phosphorylation of Ser133 is required for CRE-mediated transcription but not for dimerization and DNA-binding activity. Phosphorylation does however increase CREB's affinity for its promoter site. Upon cell stimulation, kinases are activated by cAMP production and translocate to the nucleus where they phosphorylate CREB at Ser133. CREB is almost exclusively nuclear in both unstimulated and stimulated cells.

Nucleotide	Vector Site
(none)	Promoter
14-100	CRE Tandem Response Element
371-1584	Gaussia luciferase gene (luc+)
2110	ColE1-derived plasmid replication origin
2872-3732	Beta-lactamase gene (Ampr)
3884-4037	Synthetic (upstream) poly(A) signal
342-359	pRR-REV: 5'-GTTCCCTGCTCTCTGTCG-3'
3902-3919	pRR-FWD: 5'-TCATTACATCTGTGTGTT-3'
	CRE Response Element Sequence: TGACGTCA



Activation of CRE in mammalian cells with 2 µM isoproterenol

These conditions are recommended as guidelines only. The protocol below has been optimized for transfection of 293 cells (ATCC Catalog No. CRL-11268) in a 100 mm plate using Fugene® 6 Transfection Reagent (Roche Catalog No. 11814443001). Isoproterenol was purchased from Sigma, Catalog No. I5752. Conditions should be optimized for each cell line and each transfection reagent used and kept consistent to obtain reproducible results. Prepare the RapidReporter plasmid DNA, 1X Gaussia Lysis Buffer and Gaussia Assay Buffer with Substrate as outlined in the RapidReporter manual.

DAY 1: Plating Cells
Plating Cells at 1.7 x 106 cells/100 mm plate.
Note: This amount of cells should yield 50-80% confluence on the day of transfection. Lower confluence is required to allow enough surface area for growth during the experiment period.
1. Carefully aspirate the media from a confluent 100 mm plate and briefly wash twice with 10 mL of PBS
2. Add 1 ml trypsin and incubate for 5 minutes at 37°C in a humidified atmosphere containing 5% CO2.
3. Resuspend the cells with 9 ml of cell growth media and transfer to a sterile 50 ml conical tube.
4. Count the cells using a hemacytometer.
5. Dilute the cells to the correct density, plate 1.7 x 106 cells in 10 ml total media on 100 mm plates and incubate at 37°C in a humidified atmosphere containing 5% CO2 overnight.

DAY 2: Transient Transfection
1. Ensure that the cells from Day 1 are the correct cell density.
2. For each 100 mm plate of cells to be transfected add 34 µl FuGENE 6 Reagent to 550 µl of Opti-MEM® I Reduced Serum Medium or serum-free culture media being careful not to touch the sidewall of the microcentrifuge tube.
3. Tap to mix and incubate for 5 minutes at room temperature .
4. Add 6-11 µg of pRR vector DNA (or sterile water to mock transfected cells) to the diluted FuGENE 6 mix.

5. Tap to mix and incubate for 15 minutes at room temperature to allow DNA/transfection reagent c
Note: Do not allow undiluted FuGENE 6 Reagent to come into contact with plastic surfaces other than pipette tips. Once the FuGENE 6 Reagent is diluted, combine it with the diluted DNA (from Step 1) within 45 minutes.
6. Tap to mix the transfection mix again and add the entire mixture to each plate. Swirl and return the plate(s) to the 37°C incubator containing 5% CO2 (there is no need to remove the media or wash the cells). Allow the cells to transfect for at least 4-5 hours before re-plating.

Replating onto 96-well plates:
We recommend Costar 9102 strip-well plates
1. Carefully aspirate the media from the 100 mm plate and wash twice with 10 ml of sterile PBS.
Note: Add the PBS slowly to the side of the plate to minimize disturbing the cells.
2. Add 1 ml trypsin and incubate for 5 minutes at 37°C in a humidified atmosphere containing 5% CO2.
3. Resuspend the trypsinized cells with 9 ml of cell growth media and transfer to a 50 ml conical tube for counting.
4. Count cells using a hemacytometer to determine cells per ml of media. Dilute cells with cell growth media to 30,000 cells per 190 µl of media (depending on the cell line used, optimal plating density will vary).
5. Transfer the transiently transfected cells to a sterile plastic reservoir and add 190 µl to each well using a muliti-channel pipette.
6. Return 96-well plates to the 37°C incubator (humidified atmosphere containing 5% CO2) for overnight (at least 16 hours).

Day 3: Stimulation, Lysis and Measurement:
1. Prepare a 20X stock of PMA (400 ng/ml) in cell growth media.
2. Add the stimulator to a sterile plastic reservoir
3. Using a multichannel pipette add 10 µl of 400 ng/ml PMA to each well designated for stimulation.
Note: Negative pRR transfected control wells do not receive any transcriptional stimulator; however, the same stimulation treatment is done with mock transfected cells.
4. The plate is returned to the 37°C incubator for the required amount of time.

Lysis and Measurement:
Using 1X Lysis Buffer and Assay Buffer prepared as described in the RapidReporter manual.
1. After the desired times post-stimulation, for example: 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, or 15 hours, remove media from 96-well culture plate and add 20 µl of 1X Lysis Buffer perwell.
2. Incubate at room temperature for at least 30 minutes (minimum lysis times and volumes may vary between cell types, 3 hours is the maximum time form cell lysis). Samples may be stored at room temperature for several hours or at < -20°C for longer-term storage if desired.
Note: We have noted that compared to other luciferases, Gaussia luciferase-containing lysates are more resistant to prolonged storage and freeze-thaw cycles.
3. Measure flash luminescence using a luminometer set to inject 60 µl of 1X Assay Buffer (with substrate) per well (ideally followed by a 1 second shaking) and an immediate read. If an injecting luminometer is not available, manually add 1X Assay Buffer (with substrate) at 60 µl per well by columns using a multichannel pipette, and immediately read in a luminometer.
Note: We use the Tecan Genios luminometer set at the following measurement parameters:
a. Set the gain to 150 and the temperature to maintain at 27°C.
b. Set the machine to measure luminescence and allow for stabilization prior to reading plates (read without plate prior to adding substrate/assay buffer).
c. Choose the correct plate map and read by column (i.e. A1, B1…).
Data Analysis:
Calculate the fold of induction of stimulated versus unstimulated cells from transfected and mock-transfected cells. Graph fold of induction versus time.

References:
1. Montminy M.R. et al. (1996) PNAS. 83(18): 6682-6686.

Storage
Luciferase Assays provide 100 or 1000 reactions of 5X Lysis and 1X Assay Buffers and Gaussia Substrate. Vector kits provide 10 µg of either empty or pre-made vector. Assays sold with vector contain the appropriate vector, 100 reactions of Lysis and Assay Buffers and Gaussia Substrate, as well as a positive control vector driven by the EF1α promoter. Store at -20°C, see manual for details. All reagents are guaranteed stable for 6 months when stored properly.