

RapidReporter® pRR-High-NFκB

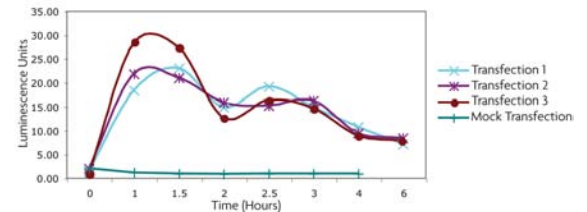
Catalog No: 33009, 33010

Background:

Transcription factors such as NFκB (nuclear factor κB) are involved in regulating gene expression through binding with the promoter region upstream of the gene to either facilitate or inhibit transcription. As described by Bauerle and Bachwal1, NFκB is specifically implicated in the regulation of many genes that code for mediators of the immune, acute phase and inflammatory responses. NFκB can be activated by a number of stimuli, including inflammatory cytokines TNFα or IL-1β, and drugs such as phorbol myristate acetate (PMA). Both TNFα and PMA are known to affect the PKA or PKC mediated signals.

Nucleotide	Vector Site
(none)	Promoter
14-58	NFκB Tandem Response Element
329-1542	Gaussia luciferase gene (luc+)
2068	ColE1-derived plasmid replication origin
2830-3690	Beta-lactamase gene (Ampr)
3842-3995	Synthetic (upstream) poly(A) signal
300-317	pRR-REV: 5'-GTTCCCTGCTCTCTGTCG-3'
3860-3877	pRR-FWD: 5'-TCATTACATCTGTGTGTT-3'

NFκB Response Element Sequence: GGAATTTC



Activation of NFκB in mammalian cells with 200 ng/ml PMA

These conditions are recommended as guidelines only. The protocol below has been optimized for transfection of HeLa cells (ATCC Catalog No. CCL-2) in a 100 mm plate using Fugene® 6 Transfection Reagent (Roche Catalog No. 11814443001). 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 1.7×10^6 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.

- Carefully aspirate the media from a confluent 100 mm plate and briefly wash twice with 10 mL of PBS
- Add 1 ml trypsin and incubate for 5 minutes at 37°C in a humidified atmosphere containing 5% CO₂.
- Resuspend the cells with 9 ml of cell growth media and transfer to a sterile 50 ml conical tube.
- Count the cells using a hemacytometer.
- Dilute the cells to the correct density, plate 1.7×10^6 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

- Ensure that the cells from Day 1 are the correct cell density.
- 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 complexes to form.
- 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 replating.

Replating onto 96-well plates 5 hours after transfection:

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 multi-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 IFN γ (2 $\mu\text{g}/\text{ml}$) in cell growth media at 37°C.
2. Add the stimulator to a sterile plastic reservoir
3. Using a multichannel pipette add 10 μl of IFN γ (2 $\mu\text{g}/\text{ml}$) to each well designated for stimulation. This corresponds to a 100 ng/ml final concentration)

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.

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 EF1a promoter. Store at -20°C, see manual for details. All reagents are guaranteed stable for 6 months when stored properly.