9891 Irvine Center Dr. Suite 200
Irvine, CA 92618
United States
Tel: 1.800.831.1518
Fax: 1.800.831.1518
Email: sale@westbioscience.com

Data Sheet

CRE/CREB Reporter Assay Kit (cAMP/PKA Cell Signaling Pathway)
Catalog #: w70622

Background

The main role of the cAMP response element, or CRE, is mediating the effects of Protein Kinase A (PKA) by way of transcription. It is the main binding site of CREB and is responsible for its activation. CRE is at the focus of many extracellular and intracellular signaling pathways, including cAMP, calcium, GPCR (G-protein coupled receptors) and neurotrophins. The cAMP/PKA signaling pathway is critical to numerous life processes and living organisms. In the cAMP/PKA signaling pathway, CREB is activated via phosphorylation of PKA and binds to CRE with a general motif of 5'-TGACGTCA-3'. Since CRE is a modulator of the cAMP/PKA signaling pathway, it allows the effects of various inhibitors to be studied.

Description

The CRE/CREB Reporter kit is designed for monitoring the activity of the cAMP/PKA signaling pathway in cultured cells. The kit contains transfection-ready CRE luciferase reporter. This reporter contains the firefly luciferase gene under the control of multimerized cAMP response element (CRE) located upstream of a minimal promoter. Elevation of the intracellular cAMP level activates cAMP response element binding protein (CREB) to bind CRE and induces the expression of luciferase.

The CRE reporter is premixed with constitutively-expressing Renilla (sea pansy) luciferase vector that serves as an internal control for transfection efficiency. The kit also includes a non-inducible firefly luciferase vector premixed with the constitutively-expressing Renilla luciferase vector as a negative control. The non-inducible luciferase vector contains the firefly luciferase gene under the control of a minimal promoter, but without any additional response elements. The negative control is critical to determining pathway-specific effects and background luciferase activity.

Applications

- Monitor cAMP/PKA signaling pathway activity.
- Screen activators or inhibitors of PKA or cAMP/PKA pathway components.
- Study effects of RNAi or gene overexpression on the activity of the cAMP/PKA pathway.

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Components

Component	Specification	Amount	Storage
Reporter (Component A)	CRE luciferase reporter vector + constitutively expressing Renilla luciferase vector	500 µl (60 ng DNA/µl)	-20°C
Negative Control Reporter (Component B)	Non-inducible luciferase vector + constitutively expressing Renilla luciferase vector	500 µl (60 ng DNA/µl)	-20°C

These vectors are suitable for transient transfection. They are NOT SUITABLE for transformation and amplification in bacteria.

Materials Required but Not Supplied

- Mammalian cell line and appropriate cell culture medium
- 96-well tissue culture plate or 96-well tissue culture treated white clear-bottom assay plate (Corning # 3610)
- Transfection reagent for mammalian cell line [We use Lipofectamine™ 2000 (Invitrogen # 11668027). However, other transfection reagents work equally well.]
- Opti-MEM I Reduced Serum Medium (Invitrogen #31985-062)
- Dual luciferase assay system:
  Dual-Glo® Luciferase Assay System (Promega #E2920): This system assays cells directly in growth medium. It can be used with any luminometer. Automated injectors are not required.
  OR
  Dual-Luciferase® Reporter Assay System (Promega #E1910): This system requires a cell lysis step. It is designed for a luminometer with automated injectors.
- Luminometer

Generalized Transfection and Assay Protocols

The following procedure is designed to transfect the reporter to HEK293 cells using Lipofectamine 2000 in a 96-well format. To transfect cells in different tissue culture formats, adjust the amounts of reagents and cell number in proportion to the relative surface area. If using a transfection reagent other than Lipofectamine 2000, follow the manufacture's transfection protocol. Transfection condition should be optimized according to the cell type and study requirement.

All amounts and volumes in the following protocol are provided on a per well basis.

1. One day before transfection, seed HEK293 cells at a density of 30,000 cells per well in 100 µl of growth medium so that cells will be 90% confluent at the time of transfection.

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2. The next day, for each well, prepare complexes as follows:

   a. Dilute DNA mixtures in 15 µl of Opti-MEM I medium (antibiotic-free). Mix gently. Depending upon the experimental design, the DNA mixtures may be any of following combinations:

      - **1 µl of Reporter** (component A); in this experiment, the control transfection is **1 µl of Negative Control Reporter** (component B).
      - **1 µl of Reporter** (component A) + experimental vector expressing gene of interest; in this experiment, the control transfection is: **1 µl of Reporte**r (component A) + negative control expression vector, **1 µl of Negative Control Reporter** (component B) + experimental vector expressing gene of interest, and **1 µl of Negative Control Reporter** (component B) + negative control expression vector.
      - **1 µl of Reporter** (component A) + specific siRNA; in this experiment, the control transfection is: **1 µl of Reporte**r (component A) + negative control siRNA, **1 µl of Negative Control Reporter** (component B) + specific siRNA, and **1 µl of Negative Control Reporter** (component B) + negative control siRNA.

   Note: We recommend setting up at least triplicate for each assay condition, and prepare transfection cocktail for multiple wells to minimize pipetting errors.

   b. Mix Lipofectamine 2000 gently before use, then dilute 0.35 µl of Lipofectamine 2000 in 15 µl of Opti-MEM I medium (antibiotic-free). Incubate for 5 minutes at room temperature.

   Note: Prepare this dilution cocktail in volumes sufficient for the whole experiment.

   c. After the 5 minute incubation, combine the diluted DNA with diluted Lipofectamine 2000. Mix gently and incubate for 25 minutes at room temperature.

3. Add the 30 µl of complexes to each well containing cells and medium. Mix gently by tapping the plate.

4. Incubate cells at 37℃ in a CO_2 incubator. Approximately 24-48 hours after transfection, perform dual luciferase assay following manufacturer's protocol.
   To study the effect of activators / inhibitors on the cAMP pathway, treat the cells with test activator/inhibitor after ~6 hours or ~ 24 hours of transfection. Perform dual luciferase assay ~24-48 hours after transfection.

Case Studies

A. Sample protocol to determine the dose response of HEK293 cells transfected with CRE reporter to Forskolin, a compound that raises the intracellular level of cAMP.

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1. One day before transfection, seed HEK293 cells at a density of 30,000 cells per well into white clear-bottom 96-well plate in 100 µl of growth medium (MEM/EBSS (Hyclone #SH30024.01) + 10% FBS, 1% non-essential amino acid, 1 mM Na-pyruvate + 1% Pen/Strep). Incubate cells at 37° in a CO_2 incubator for overnight.

2. The next day, transfect 1 µl of CRE luciferase reporter (component A) into cells following the procedure in **Generalized Transfection and Assay Protocols.**

3. After ~22-24 hours of transfection, add threefold serial dilution of forskolin in 50 µl of growth medium to stimulated wells. Add 50µl of growth medium with 0.1% DMSO to unstimulated control wells; add 50µl of growth medium to cell-free control wells (for determining background luminescence). Set up each treatment in at least triplicate.

4. Incubate at 37° in a CO_2 incubator for 5-6 hours.

5. Perform dual luciferase assay using Dual-Glo® Luciferase Assay System (Promega #E2920): Add 50 µl of Luciferase reagent per well and rock at room temperature for ~15 minutes and measure firefly luminescence using a luminometer. Add 50 µl of Stop & Glo™ reagent per well. Rock gently at room temperature for ~15 minutes and measure Renilla luminescence.

6. To obtain the normalized luciferase activity for CRE reporter, subtract background luminescence, then calculate the ratio of firefly luminescence from the CRE reporter to Renilla luminescence from the control Renilla luciferase vector.

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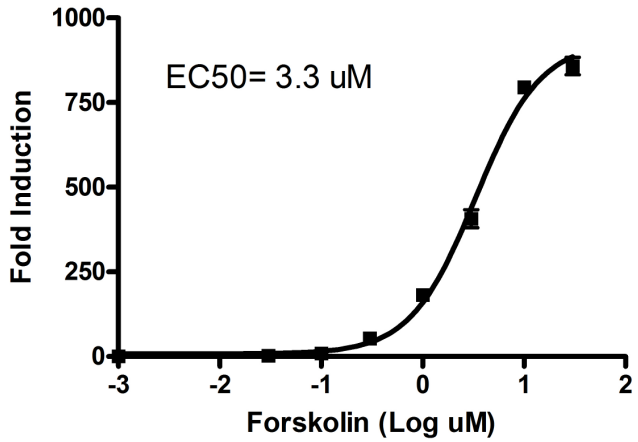
Figure 1. Dose response of CRE reporter activity to forskolin. The results were shown as fold induction of normalized CRE luciferase reporter activity. Fold induction was determined by comparing values against the mean value for control cells without forskolin treatment.

The EC50 of Forskolin is ~3.3 μM.



B. Use CRE reporter to study Phosphodiesterase (PDE) activity:
PDEs regulate the intracellular levels of cAMP and cGMP by hydrolyzing cAMP and cGMP to their inactive 5' monophosphates. When cells transiently transfected with CRE reporter are activated by forskolin, the intracellular level of cAMP is upregulated, which induces the expression of CRE luciferase reporter. However, when cells are co-transfected with PDE expression vector and CRE reporter, the level of forskolin-induced cAMP is reduced, resulting in lower expression level of luciferase. When cells are treated with PDE inhibitor to inhibit PDE activity, cAMP level is restored, resulting in higher luciferase activity.

Sample protocol to determine the effect of antagonists of PDE4D on CRE reporter activity in HEK293 cells.

1. One day before transfection, seed HEK293 cells at a density of 30,000 cells per well into white clear-bottom 96-well plate in 100 μl of growth medium. Incubate cells at 37° in a CO_2 incubator for overnight.

2. Next day, transfect 1 μl of CRE luciferase reporter (component A) with PDE4D7 expression vector or control expression vector into cells following the procedure in **Generalized Transfection and Assay Protocols.**

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3. After ~6 hours of transfection, treat transfected cells with PDE4D inhibitor, Rolipram, in 50 µl of fresh growth medium. Incubate cells overnight at 37° in a CO_2 incubator.

4. After ~22-24 hours of transfection, add forskolin (final concentration 10 µM) in 5 µl of growth medium to stimulated wells (cells treated with forskolin, with or without rolipram). Add 5 µl of growth medium with 1% DMSO to the unstimulated control wells (cells without rolipram and forskolin for determining the basal activity). Add 55 µl of growth medium to cell-free control wells (for determining background luminescence). Set up each treatment in at least triplicate.

5. Incubate at 37° in a CO_2 incubator for 5-6 hours.

6. Perform dual luciferase assay using Dual-Glo® Luciferase Assay System (Promega #E2920): Add 55 µl of Luciferase reagent per well. Rock gently at room temperature for ~15 minutes, then measure firefly luminescence using a luminometer. Add 55 µl of Stop & Glo™ reagent per well and rock at room temperature for ~15 minutes, then measure Renilla luminescence.

7. To obtain the normalized luciferase activity of CRE reporter, subtract background luminescence then calculate the ratio of firefly luminescence from the CRE reporter to Renilla luminescence from the control Renilla luciferase vector.

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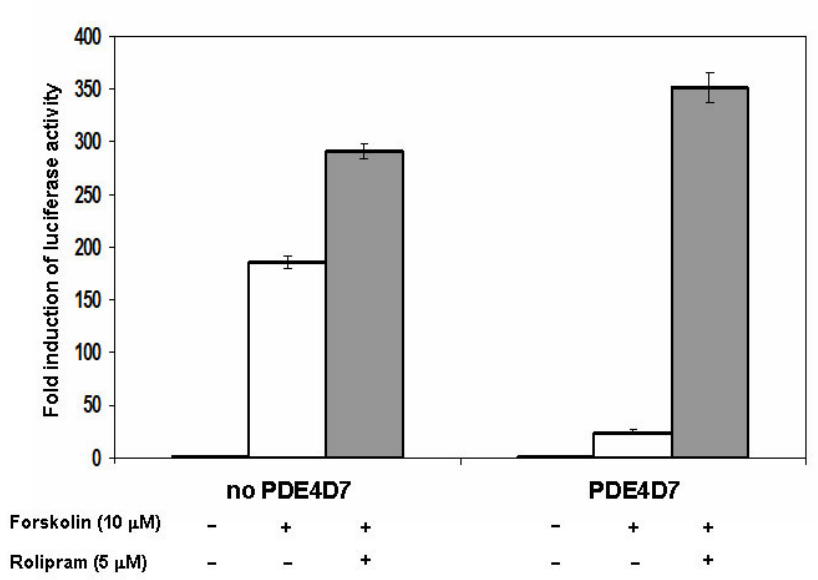
Figure 2. PDE4D7 reduces the level of cAMP following forskolin stimulation. This effect is reversed by Rolipram, a PDE4 inhibitor. The data are shown as fold induction of normalized CRE luciferase reporter activity. Fold induction was determined by comparing values against the mean value for control cells without forskolin treatment.



References:

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