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Data Sheet

The Transfection Collection™ – CRE/CREB Transient Pack (cAMP/PKA Cell Signaling Pathway)

Catalog #: 79267

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 Transient Pack is designed to provide the tools necessary for transiently transfecting and monitoring the activity of the cAMP/PKA signaling pathway in cultured cells. The kit contains transfection-ready vectors containing firefly luciferase as a cAMP/PKA pathway-responsive reporter and constitutively expressing Renilla luciferase as a transfection control. It also includes the Dual Luciferase detection reagents to detect both luciferase activities and specialized medium for growing and assaying HEK293 cells.

The key to the CRE/CREB Transient Pack is the CRE/CREB luciferase reporter vector, which is a cAMP/PKA Cell Signaling Pathway-responsive reporter. This reporter contains the firefly luciferase gene under the control of multimerized cAMP response elements (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.

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Additionally, the pack includes cell culture medium (BPS Medium 1) that has been optimized for use with HEK293 cells*. BPS Medium 1 includes MEM medium, 10% fetal bovine serum, 1% non-essential amino acids, sodium pyruvate, and 1% Pen/Strep. Finally, the pack provides the Dual Luciferase (Firefly-Renilla) Assay System. These luciferase reagents provide highly sensitive, stable detection of firefly luciferase activity and Renilla luciferase activity. The dual luciferase reagents can be used directly in cells in growth medium, and can be detected with any luminometer; automated injectors are not required.

* Other mammalian cell lines may also be used, but an alternate cell culture medium may be required for optimal cell growth.

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.

Components

Component	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
Firefly Luciferase Reagent Buffer	10 ml	-20°C
Firefly Luciferase Reagent Substrate (100x)	100 µl	-20°C *Protect from light*
Renilla Luciferase Reagent Buffer	10 ml	Room Temp.
Renilla Luciferase Reagent substrate (100x)	100 µl	-20°C *Protect from light*
BPS Medium 1	100 ml	+4°C

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

Materials Required but Not Supplied

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- HEK293 cells. Other mammalian cell lines may also be used, but an alternate cell culture medium may be required for optimal cell growth.
- 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)
- Luminometer

(Optional)
- Forskolin (BPS Bioscience, #27067) in DMSO or other adenylate cyclase activator
- S- (+)-Rolipram (BPS Bioscience, #27648-2) in DMSO or other PDE inhibitor

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 manufacturer's transfection protocol. Transfection condition should be optimized according to the cell type and study requirements.

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 BPS Medium 1 so that cells will be 90% confluent at the time of transfection.

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**

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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. Carefully remove and discard 30 µl of media from each of the wells of cell culture, taking care not to disturb the cells or touch the bottom of the well with the pipet tip. Add the 30 µl of the complexes to each well containing 70 µl cells and medium. Mix gently by tapping the plate.

4. Incubate cells at 37°C in a CO_2 incubator. Approximately 24-48 hours after transfection, perform the Dual Luciferase Assay System (below).

   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.

Dual Luciferase Assay Procedure

1. Thaw Firefly Luciferase Reagent Buffer by placing the reagent in a room temperature water bath. Equilibrate the buffer to room temperature and mix well before use. Note: It is important that the Firefly Luciferase Reagent Buffer be at room temperature before use.
2. Calculate the amount of Firefly Luciferase Assay Working solution needed for the experiment (Firefly Luciferase Reagent Buffer + Firefly Luciferase Reagent Substrate). Immediately prior to performing the experiment, prepare the Firefly Luciferase Assay Working Solution by diluting Firefly Luciferase Reagent Substrate into Firefly Luciferase Reagent Buffer at a 1:100 ratio and mix well. Avoid exposing to excessive light. Only use enough of each component for the experiment, remaining Firefly Luciferase Reagent Buffer and Firefly Luciferase Reagent Substrate should be stored separately at -20°C.
3. Remove multi-well plate containing mammalian cells from incubator. Note: plates must be compatible with luminescence measurement by luminometer being used.

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4. Add equal volume of Firefly Luciferase Assay Working Solution (step 2) to the culture medium in each well. Example: 96-well plate with 100 µl of culture medium requires 100 µl of Firefly Luciferase Assay Working Solution per well.

   Gently rock the plates for ~15 minutes at room temperature. Measure firefly luminescence using a luminometer. The signal under these conditions will be stable for more than 2 hours at room temperature. For maximal light intensity, measure samples within 1 hour of reagent addition.

5. Calculate the amount of Renilla Luciferase Assay Working Solution needed for the experiment (Renilla Luciferase Reagent Buffer + Renilla Luciferase Reagent Substrate). Prepare the Renilla Luciferase Assay Working Solution by diluting Renilla Luciferase Reagent Substrate into Renilla Luciferase Reagent Buffer at a 1:100 ratio and mix well. Avoid exposing to excessive heat or light. Only use enough of each component for the experiment.
6. Add equal volume of Renilla Luciferase Assay Working Solution (step 5) to each well. Example: 96-well plate with 100 µl of culture medium + 100 µl Firefly Luciferase Reagent requires 100 µl of Renilla Luciferase Assay Working Solution per well.
7. Gently rock the plates for ~1 minute at room temperature. Measure Renilla luminescence using a luminometer.
8. Data analysis: subtract background (wells with medium and luciferase reagent only) from all the readings. To obtain the normalized luciferase activity for the CRE reporter, subtract the background luminescence, then calculate the ratio of firefly luminescence from CRE reporter to Renilla luminescence from the control Renilla luciferase vector. To determine the fold induction, divide the value for the treated cells by the value for the untreated cells.

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.

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 BPS Medium 1. Incubate cells at 37°C 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 BPS Medium 1 to stimulated wells. Add 50µl of BPS Medium 1 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.

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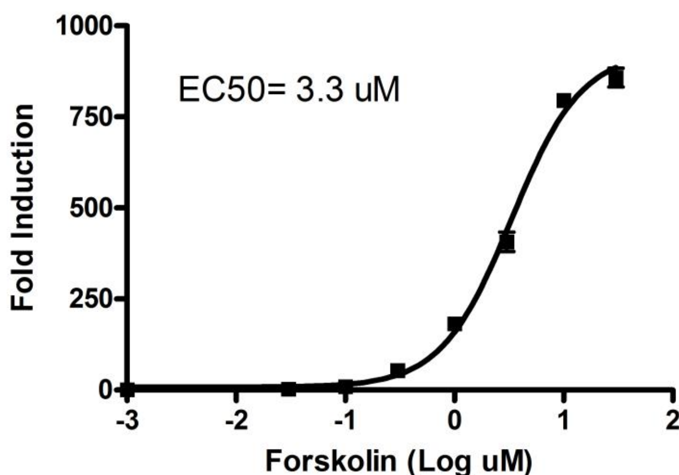
4. Incubate at 37°C in a CO_2 incubator for 5-6 hours.

5. Perform dual luciferase assay as described above in Dual Luciferase Assay Procedure. Briefly, dilute 100x Firefly Luciferase Reagent Substrate into Firefly Luciferase Reagent Buffer that has been equilibrated to room temperature . Add 50 µl of Firefly Luciferase reagent per well and rock at room temperature for ~15 minutes, then measure firefly luminescence using a luminometer. Dilute 100x Renilla Luciferase Reagent Substrate into Renilla Luciferase Reagent Buffer . Add 50 µl of Renilla Luciferase reagent per well, rock at room temperature for ~1 minute 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.

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

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Using 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.

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 BPS Medium 1. Incubate cells at 37°C 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.**

3. After ~6 hours of transfection, treat transfected cells with PDE4D inhibitor, Rolipram, in 50 µl of fresh BPS Medium 1. Incubate cells overnight at 37°C in a CO_2 incubator.

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

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

6. Perform dual luciferase assay as described above in Dual Luciferase Assay Procedure. Briefly, dilute 100x Firefly Luciferase Reagent Substrate into Firefly Luciferase Reagent Buffer that has been equilibrated to room temperature. Add 55 µl of Firefly Luciferase reagent per well and rock at room temperature for ~15 minutes, then measure firefly luminescence using a luminometer. Dilute 100x Renilla Luciferase Reagent Substrate into Renilla Luciferase Reagent Buffer . Add 55 µl of Renilla Luciferase reagent per well, rock at room temperature for ~1 minute and 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.

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

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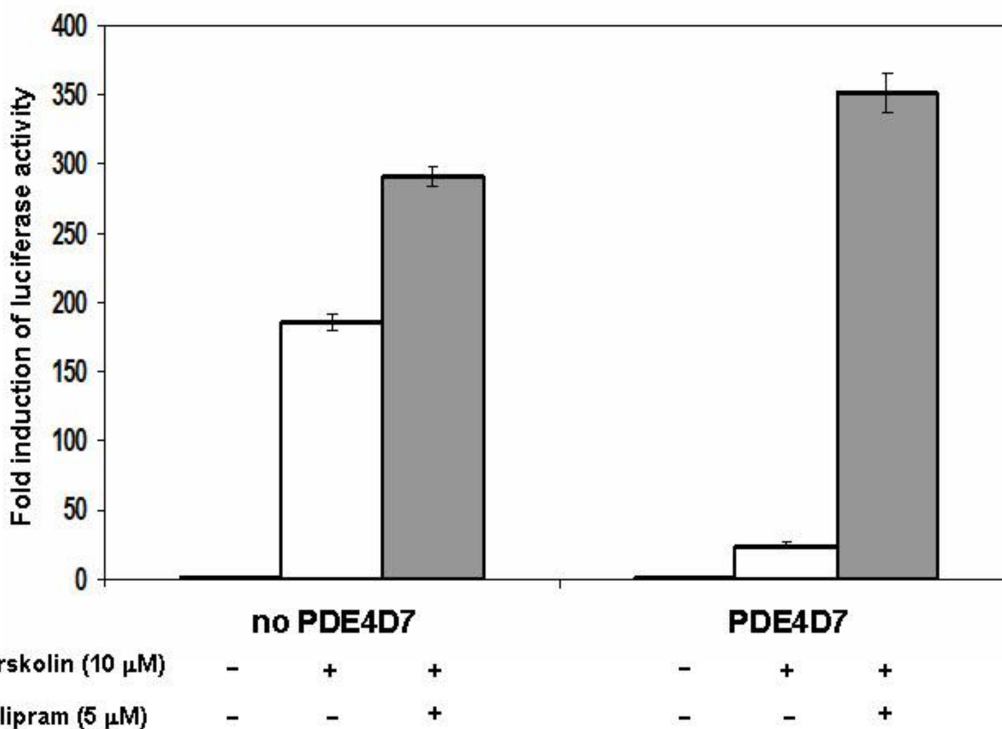
normalized CRE luciferase reporter activity. Fold induction was determined by comparing values against the mean value for control cells without forskolin treatment.



References:

1. Montminy, M.R. *et al.* (1987) Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature* **328(6126):**175-178.
2. Fan Chung, K. (2006) Phosphodiesterase inhibitors in airways disease. *Eur. J. Pharmacol.* **533(1-3):**110-117.
3. Malik, R. *et al.* (2008) Cloning, stable expression of human phosphodiesterase 7A and development of an assay for screening of PDE7 selective inhibitors. *Appl. Microbiol. Biotechnol.* **77 (5):** 1167-1173.

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Refills

Product	Catalog #	Size
CRE/CREB Reporter Kit (cAMP/PKA Signaling Pathway)	60611	500 rxns.
Dual Luciferase (Firefly-Renilla) Assay System	60683-1	10 ml
Dual Luciferase (Firefly-Renilla) Assay System	60683-2	100 ml
Dual Luciferase (Firefly-Renilla) Assay System	60683-3	1 L
BPS Medium 1	79259	100 ml

Related Products

Product	Catalog #	Size
CRE/CREB Reporter (Luc) – HEK293 Cell Line	60515	2 vials
CREBBP (KAT3A), His-tag	31119	100 µg
CREBBP (KAT3A), GST-tag	31128	100 µg
CREBBP TR-FRET Assay Kit	32619	384 rxns.
PDE Assay Kit	60300	96 rxns.
PDE4D2 Assay Kit	60707	96 rxns.
PDE4D3 Assay Kit	60701	96 rxns.
PDE4D7 Assay Kit	60708	96 rxns.

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