6405 Mira Mesa Blvd Ste 100
San Diego, CA 92121
Tel: 1.858.202.1401
Fax: 1.858.481.8694
Email: support@bpsbioscience.com

Data Sheet

The Transfection Collection™ – PDE4D Transient Pack

Catalog #79286

Background

Phosphodiesterases (PDEs) play an important role in the dynamic regulation of cAMP and cGMP signaling. PDE4D has 3',5'-cyclic-AMP phosphodiesterase activity and degrades cAMP. Inhibition of PDE4D activity by its inhibitors leads to an elevated intracellular level of cAMP. The PDE4D gene encodes at least 9 different isoforms, and has been linked to stroke, asthma, arrhythmia, and cardiac myopathy, making it an important therapeutic target.

Description

The PDE4D *Transient Pack* is designed to provide the tools necessary for transiently transfecting and for screening inhibitors of PDE4D7 in cultured HEK293 cells.

The assay is based on transfecting cells with the CRE luciferase reporter. CRE reporter contains the firefly luciferase gene under the control of cAMP response element (CRE). Elevation of intracellular cAMP activates CRE binding protein (CREB) to bind CRE and induce the expression of luciferase. Forskolin is commonly used to raise the intracellular level of cAMP in cell physiology studies. 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 PDE4D7 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 PDE4D inhibitor to inhibit PDE4D7 activity, cAMP level is restored, resulting in higher luciferase activity.

The PDE4D *Transient Pack* contains transfection-ready CRE luciferase reporter. This reporter contains transfection-ready vectors for PDE4D7 expression and a CRE vector containing firefly luciferase as a cAMP pathway-responsive reporter and constitutively expressing Renilla luciferase as a transfection control. It also includes the TWO-Step Luciferase detection reagents to detect both luciferase activities and specialized medium for growing and assaying HEK293 cells.

The key to the PDE4D Cell-Based Activity Reporter Detection Valuepack is the CRE 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. The vector is premixed with constitutively-expressing Renilla (sea pansy) luciferase vector that serves as an internal control for transfection efficiency. The pack also

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includes the PDE4D7 expression vector, and the adenylate cyclase activator, forskolin. Additionally, the pack includes cell culture medium (BPS Medium 1) that has been optimized for use with HEK293 and HeLa 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 TWO-Step Luciferase (Firefly & Renilla) Assay System. These luciferase reagents provide highly sensitive, stable detection of firefly luciferase activity and Renilla luciferase activity. The TWO-step luciferase reagents can be used directly in cells in growth medium, and can be detected with any luminometer; automated injectors are not required.

*Note: the kit may be used with other cell lines than HEK293 or HeLa, but an alternate cell culture medium may be required for optimal cell growth

Applications

- Screen PDE4D inhibitors for drug discovery.
- Monitor cAMP/PDE4D signaling pathway activity

Components

Component	Amount	Storage
Reporter (Component A) CRE luciferase reporter vector* + constitutively expressing Renilla luciferase vector*	500 µl (60 ng DNA/ µl)	-20°C
PDE4D7 expression vector (Component B)	250 µl (40 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 designed for transient transfection. They are NOT suitable for transformation and amplification in bacteria.

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Materials Required but Not Supplied

- 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-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)
- S- (+)-Rolipram (BPS Bioscience, #27648-2) in DMSO or other PDE inhibitor
- Luminometer
- Forskolin (BPS Bioscience #27067) or other reference compound

Generalized Transfection and Assay Protocols

The following procedure is designed for transfection of 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 recommended 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 into a white clear-bottom 96-well plate in 100 µl of BPS Medium 1 so that cells will be 90% confluent at the time of transfection.

2. The next day, transiently transfect the cells with CRE reporter and PDE4D7 expression vectors. For each well, prepare complexes as follows:
   a. Dilute 1 µl of Reporter (component A) and 0.5 µl of PDE4D7 expression vector (component B) in 15 µl of Opti-MEM I medium (antibiotic-free). Mix gently.
   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.
   c. After the 5 minute incubation, combine the diluted DNA with diluted Lipofectamine 2000. Mix gently and incubate for 25 minutes at room temperature.

Note: we recommend setting up the assay in at least triplicate for each treatment. To minimize pipetting errors, prepare a master mix of sufficient transfection cocktail for multiple wells.

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.

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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 for 6 hours.

5. After ~6 hours, remove cell medium from transfected cells and replace with 50 µl of fresh BPS Medium 1 containing PDE4D inhibitor. The final DMSO concentration should not exceed 0.3%. Incubate cells overnight at 37°C in a CO_2 incubator.

6. After ~22-24 hours, add forskolin (final concentration 10 µM) in 5 µl of BPS Medium 1 to stimulated wells (cells treated with forskolin, with or without inhibitor). Add 5 µl of BPS Medium 1 with 1% DMSO to the unstimulated control wells (cells without inhibitor 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.

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

8. Perform TWO-step luciferase assay System (below).

TWO-Step 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.

4. Add an 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.

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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 of CRE reporter, calculate the ratio of firefly luminescence from the CRE reporter to Renilla luminescence from the control Renilla luciferase vector.

Figure 1. 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.

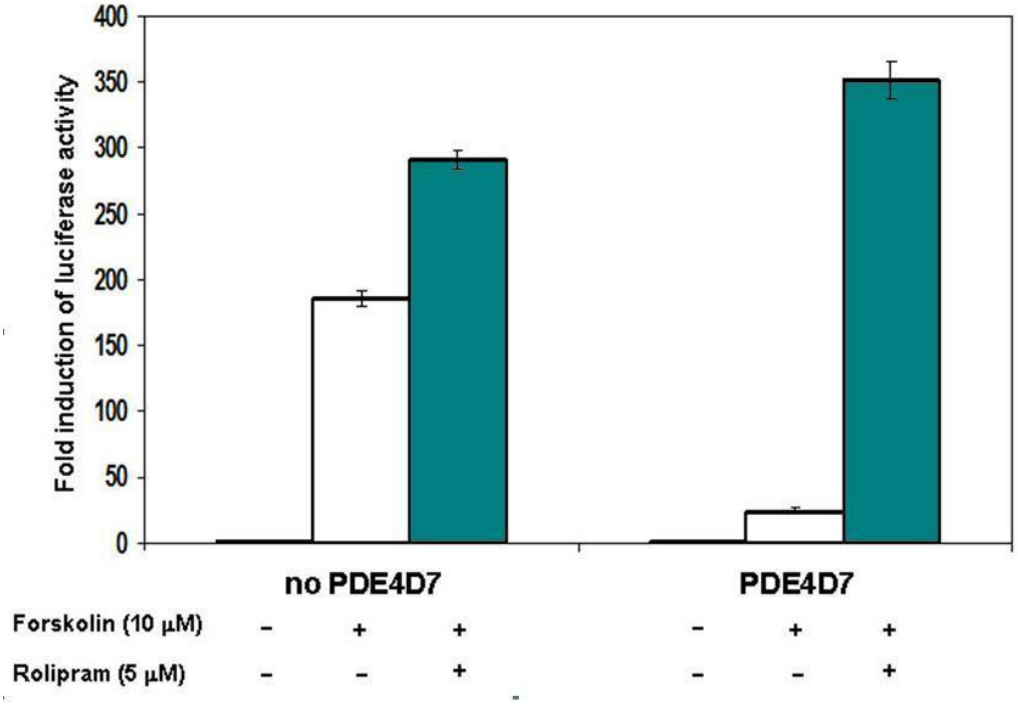

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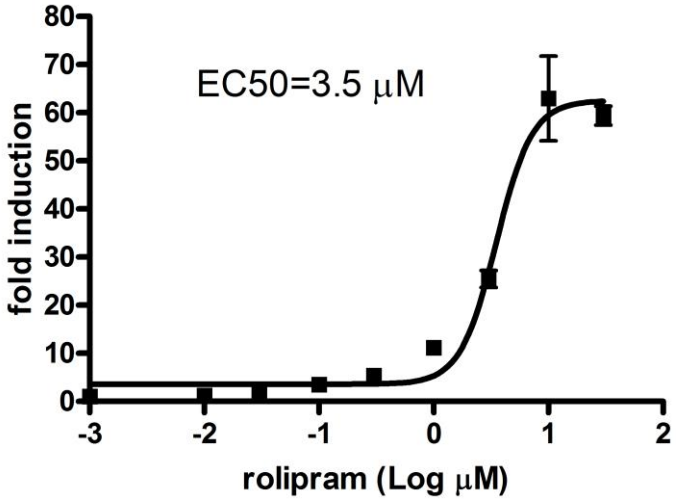
Figure 2. Rolipram dose response in PDE4D7-transfected HEK293.
The results were shown as fold induction of CRE reporter activity. Fold induction was determined by comparing values against the mean value for cells stimulated with forskolin in the absence of rolipram. The inhibition of PDE4D7 in cells induces the luminescence, so the inhibitory effects of the compounds on PDE4D7 activity is expressed as EC_{50}. The EC_{50} of rolipram is ~ 3.5 μM.



References

1. Montminy, MR *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

Refills

Product Name	Catalog #	Size
PDE4D Cell-Based Reporter Assay Kit	60505	500 rxns.
BPS Medium 1	79259	100 ml
TWO-Step Luciferase (Firefly & Renilla) Assay System	60683-1	10 mL
TWO-Step Luciferase (Firefly & Renilla) Assay System	60683-2	100 mL
TWO-Step Luciferase (Firefly & Renilla) Assay System	60683-3	1 L
Forskolin	27067	10 mg
Forskolin	27068	50 mg

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Related Products

Product	Catalog #	Size
PDE Assay Kit	60300	96 rxns.
PDE4D2 Assay Kit	60707	96 rxns.
PDE4D3 Assay Kit	60701	96 rxns.
PDE4D7 Assay Kit	60708	96 rxns.
PDE4D2, GST-tag	60048	5 µg
PDE4D3, GST-tag	60046	5 µg
PDE4D7, GST-tag	60047	5 µg