EP_4 Receptor (rat) Reporter Assay Kit

Item No. 600350

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	100 Tests Quantity/Size	Storage
600351	EP_4 Receptor (rat) Reverse Transfection Strip Plate	1 plate	-20°C
600341	EP Receptor Assay Prostaglandin E_2 Positive Control	1 vial/20 µl	-20°C
600183	SEAP Substrate (Luminescence)	1 vial/15 ml	4°C
700029	96-Well Solid Plate (white)	3 plates	RT
400012	96-Well Cover Sheet	3 covers	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

Remove the Prostaglandin E_2 Positive Control from the kit and store at -20°C. Store the EP_4 Receptor Reverse Transfection Strip Plate at -20°C. The SEAP Substrate should be stored at 4°C. The kit will perform as specified if used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. HEK293T cells; available from ATCC. Other cell lines with little or no endogenous expression of EP receptors may be used, but optimization may be required. HEK293 cells may have high levels of endogenous activity and should be used with caution
2. Culture medium used for maintenance of the cells (DMEM)
3. Reduced serum medium for plating cells on the EP_4 Receptor (rat) Reverse Transfection Strip Plate and stimulation, such as UltraMEM™ Reduced Serum Medium (Lonza) or Opti-MEM™ Reduced Serum Medium (Thermo Fisher) supplemented with 1X penicillin-streptomycin
4. Fetal bovine serum (FBS)
5. A plate reader capable of measuring luminescence
6. Adjustable pipettes and a repeating pipettor
7. An incubator set at 65°C
8. Penicillin-Streptomycin (100X)

INTRODUCTION

Background

Prostaglandin E_2 (PGE_2), one of the most important biologically active prostanoids, exerts its actions through binding to four distinct G protein-coupled receptors (GPCRs). These PGE_2 receptor subtypes, EP_1, EP_2, EP_3, and EP_4, exhibit differences in signal transduction mechanisms, tissue localization, and regulation of expression.[1] EP_4 receptors are highly expressed in the intestine, but are found in lower levels in the lung, kidney, thymus, uterus, and brain.[2,3] The receptors are coupled to Gs to stimulate the cAMP second messenger signal transduction pathway. In addition, EP_4 couples to phosphatidylinositol 3-kinase, probably *via* G_i to mediate cell survival.[4] EP_4 receptors play important roles in relaxation of smooth muscle, gastric acid secretion, intestinal epithelial transportation, adrenal aldosterone secretion, and uterine functions.[5,6] PGE_2 regulates immunity and inflammation mainly through its receptor subtypes EP_2 and EP_4.[7,8] EP_4 receptors are predominantly expressed in human colon cancers, suggesting a role for EP_4 receptors in colorectal carcinogenesis.[9] *In vivo* studies demonstrated that activation of the EP_4 receptor was neuroprotective in excitotoxic brain injury.[10] The diverse effects of PGE_2 *via* EP_4 receptors point to the need to identify novel agonists and antagonists, both to further elucidate the function of this receptor subtype and for use as therapeutics for various diseases.

About This Assay

Cayman's Reverse Transfection Reporter Assays have overcome many of the disadvantages of other transfection approaches. In this method, a proprietary transfection complex containing DNA and an optimized mixture of lipids and proteins is evenly applied and processed on the culture surface of multi-well plates. Adherent cells, supplied by the user, are applied directly to the plate and allowed to grow in the coated wells. Using this method, the uptake of the DNA complex by the cell increases dramatically compared to solution-phase transfection, enhancing both the transfection efficiency and the co-transfection efficiency for multiple plasmids.

Cayman's EP_4 Receptor (rat) Reporter Assay Kit consists of a 96-well plate coated with a transfection complex containing DNA constructs for rat EP_4 receptor and a cAMP response element regulated Secreted Alkaline Phosphatase (SEAP) reporter (EP_4 Receptor Reverse Transfection Strip Plate). Cells grown on the transfection complex will express EP_4 at the cell surface. Binding of agonists to EP_4 initiates a signal transduction cascade resulting in expression of SEAP which is secreted into the cell culture medium. Aliquots of medium are removed at time intervals, beginning at approximately six hours, and SEAP activity is measured following addition of a luminescence-based alkaline phosphatase substrate provided in the kit. The kit is simple to use and can be easily adapted to high throughput screening for therapeutic compounds regulating the activation of EP_4. PGE_2 is included in the kit for use as a positive control. The kit provides sufficient reagents to measure SEAP activity at three time points using the white plates included.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical experimental plate will include wells with cells treated with PGE_2 provided in the kit (positive control), wells with cells treated with experimental compounds, and wells of untreated cells. We recommend that each treatment be performed at least in triplicate. If you are running a test compound curve to determine an EC_{50} value, several serial dilutions of the test compound should be included in the assay. The kit contains enough PGE_2 to run a control dose-response curve as well. Record the contents of each well on the template sheet provided on page 17.

Addition of Cells to the Reverse Transfection Plate

IMPORTANT

Before starting the experiment, dilute the 100X Penicillin-Streptomycin 1:100 in reduced serum medium used for your cells. This will be the culture medium for your experiment.

1. Remove the EP_4 Receptor Reverse Transfection Strip Plate (Item No. 600351) from the refrigerator or freezer and allow it to equilibrate to room temperature. Clean the bag with 70% alcohol before opening the bag. Place the plate in the hood and remove from the bag.

   *NOTE: If you are not using the whole plate at one time for your experiment, remove the number of strips needed, put the remaining strips back in the bag, and store in a desiccator, **protected from UV light**, at room temperature for up to a week. Alternatively, you can vacuum seal the bag and store the remaining strips at -20°C for up to two months.*

2. Seed each well of the plate at a density of 40,000-80,000 cells/well in 200 µl of reduced serum medium containing 0.5% FBS. Place the plate in a 37°C incubator with 5% CO_2 and incubate overnight.

   NOTE: HEK293T cells should be maintained in DMEM with 10% FBS. Since serum may contain agonist for EP_4 receptor, caution should be taken to avoid carry-over of maintenance medium when plating cells on the reverse transfection plate in reduced serum medium.

Cell Stimulation

1. After 16-18 hours of incubation, aspirate the culture medium from each well.
2. Add 100 µl of reduced serum medium containing Penicillin-Streptomycin to each well.
3. Prepare test compounds at 2X the desired final concentration in the above reduced serum medium containing Penicillin-Streptomycin and pipette 100 µl to the assigned wells. Wells containing untreated cells receive 100 µl of medium only. For positive controls using PGE_2, dilute the PGE_2 Positive Control (Item No. 600341) 1:500,000 in the serum-free culture medium and add 100 µl to corresponding wells. At this concentration, PGE_2 induces a 3-10 fold increase in SEAP activity, depending on the cell type and stimulation time used.

NOTE: It is recommended the assay be performed when the cells are above 50% confluent. This kit could be used to characterize antagonists by co-incubation of the experimental compound with a fixed dose of PGE_2 at approximately the EC_{50}. Antagonists should be added to the cells 30 minutes before the addition of PGE_2.

Performing the SEAP Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Before performing the assay, remove the SEAP Substrate (Luminescence) (Item No. 600183) from the refrigerator and allow to equilibrate to room temperature. *Note: Vortex the substrate vigorously prior to use.*

1. After 6-8 hours of stimulation with test compounds and PGE_2 (Item No. 600341, as a positive control), transfer the plate from the incubator to a culture hood.
2. In the culture hood, remove 10 µl of culture medium from each well to a corresponding well of a 96-Well Solid Plate (white) (Item No. 700029).
3. Cover the plate with a provided 96-Well Cover Sheet (Item No. 400012).
4. Inactivate endogenous alkaline phosphatase by heating the samples at 65°C for 30 minutes. The SEAP expressed in this assay is stable under these conditions.
5. Remove the plate from the 65°C incubator and allow to equilibrate to room temperature.
6. Add 50 µl of substrate to each well, shake briefly, and incubate the plate at room temperature for 2-5 minutes.
7. Read the plate with a plate reader capable of detecting a luminescent signal.

NOTE: The plate should be read immediately after incubation. When multiple plates are processed at the same time, the addition of substrate and reading of the plate should be done plate by plate in order to allow equal time from addition of substrate to the time the plate is read.

ANALYSIS

Calculations

Determination of EC_{50}

The term half maximal effective concentration (EC_{50}) refers to the concentration of a drug which induces a response halfway between the baseline and maximum after some specific exposure time. The dose-response curve of a typical agonist follows a sigmoidal curve with a bottom plateau (untreated cells) and a top plateau (drug saturation). See Figure 1, on page 13, for a typical PGE_2 curve.

For each compound, normalize the the Relative Luminescent Unit (RLU) results to run from 0% (no drug added) to 100% (saturating dose) by using the following formula:

$$\% \text{ Response at X Concentration} = \left[\frac{(\text{RLU at X Concentration}) - (\text{RLU of untreated cells})}{\text{Maximal RLU (saturation)} - (\text{RLU of untreated cells})} \right] \times 100$$

Graph % response versus log drug concentration. In the resulting sigmodial dose-response curve, find the best-fit value for the log EC_{50} (the concentration that gives a 50% response; the middle of the curve).

Performance Characteristics

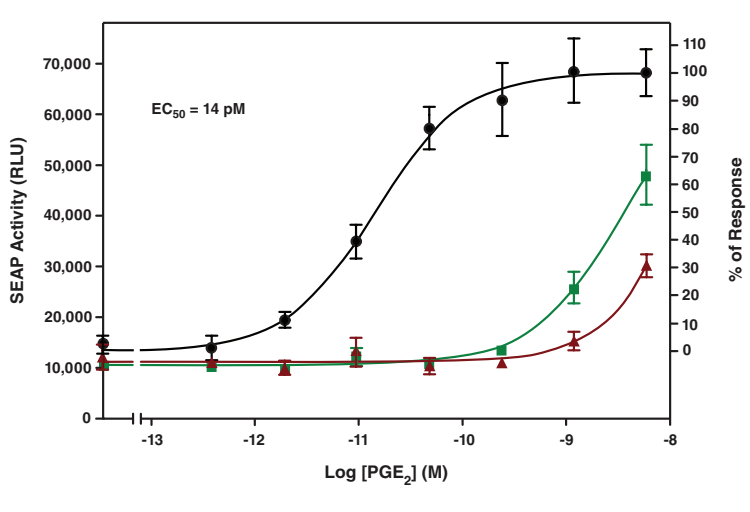

Figure 1. SEAP activity in HEK293T cells transiently-transfected with EP_4 receptor in response to PGE_2 stimulation, with or without antagonist L-161982. HEK293T cells were plated in an EP_4 Reverse Transfection Strip Plate at a density of 50,000 cells/well and incubated overnight. The next day, cells were treated with different doses of PGE_2 with or without the EP_4 antagonist L-161982 in reduced serum medium as indicated above. After seven hours of stimulation, 10 µl of culture medium was removed from each well and the SEAP activity from each sample was measured according to the protocol described on page 11. The calculated EC_{50} value from the fitted curve is 14.2 pM. Co-incubation with L-191982 led to a right shift of dose-response curve.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of replicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
Erratic response curve of compound treatments	Unequal number of cells in each well	Make sure each well contains the same number of cells
High reading in all wells	Cell density is too high or treatment was too long	Plate cells more sparsely (decrease treatment time)
No signal	A. Contamination B. Cells lost during medium change	A. Keep cells in sterile environment B. Gently aspirate supernatant; do not disturb cell layer

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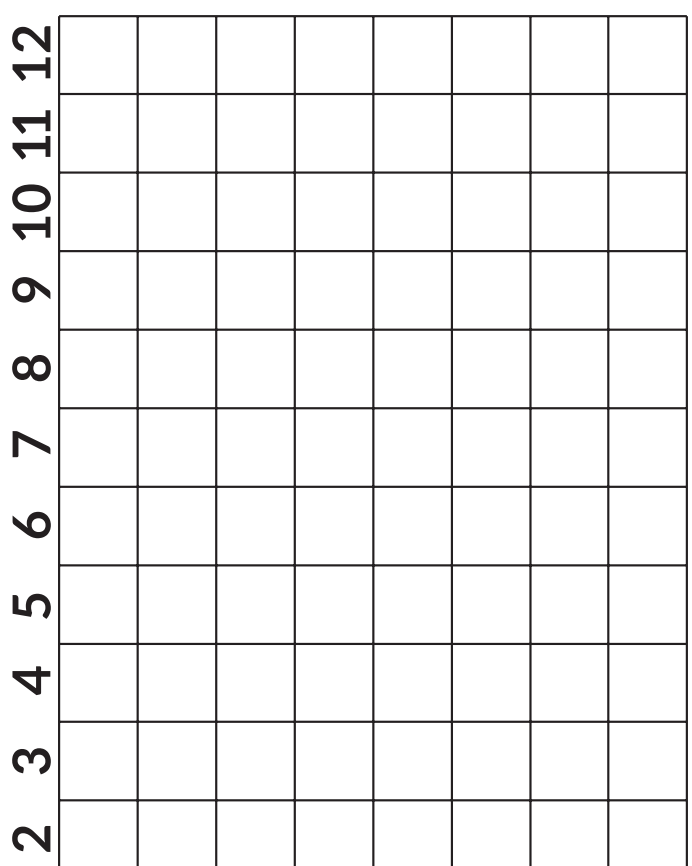

NOTES

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