Orexin 2 Receptor Reporter Assay Kit

Item No. 600250

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. For best results, remove components and store as stated below.

Item Number	Item	100 Tests Quantity/Size	Storage
600251	Orexin 2 Receptor Reverse Transfection Strip Plate	1 plate	-20°C
600242	Orexin A Positive Control	1 vial/10 µl	-80°C
600183	SEAP Substrate (Luminescence)	1 vial/15 ml	4°C
10011297	96-Well Solid Plate (black) with lid	3 plates	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

Fax: 734-971-3640

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 Orexin A Positive Control from the kit and store at -80°C (be careful to avoid repeated freeze/thaw cycles). Store the Orexin 2 Receptor Reverse Transfection Strip Plate at -20°C. The SEAP Substrate should be stored at 4°C and will be stable for at least one year. The kit will perform as specified if stored as instructed and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. HEK293 or HEK293T cells; both cell lines can be obtained from ATCC
2. Culture medium used for the cells (DMEM)
3. Fetal Bovine Serum (FBS)
4. A plate reader capable of measuring luminescence
5. Adjustable pipettes and a repeating pipettor
6. An incubator set at 65°C
7. Penicillin-Streptomycin (100X) (Gibco 15140-122)

INTRODUCTION

Background

The orexin (hypocretin) system is a critical regulator of diverse physiological actions, including control of sleep/wake cycle, feeding behavior, and reward processes as well as cardiovascular and autonomic functions.

The orexins were discovered in 1998 by two independent groups.[1,2] A common precursor gives rise to two novel hypothalamic neuropeptides, orexin A and orexin B (also known as hypocretin 1 and hypocretin 2, respectively), sharing substantial amino acid identities both with each other and with the gut hormone secretine. The precursor gene is expressed in the hypothalamus, midbrain, and brainstem, with very wide projection pattern suggesting a broad range of physiological functions.

The orexins activate two closely related G protein-coupled receptors (GPCRs), termed the orexin-1 receptors (OX1R) and orexin-2 receptors (OX2R), which are also highly conserved across mammalian species. While orexin A has equal affinity at OX1R and OX2R, orexin B has an appreciably greater affinity for OX2R.[2] The importance of orexins in the maintenance of consolidated sleep/wake states has been demonstrated by the fact that the sleep disorder narcolepsy is caused by orexin deficiency in human and animals.[3] It is also known that central administration of either orexin during the light period induces food intake in rodents, indicating that the orexin system is involved in the regulation of feeding behavior and energy homeostasis.[4] Moreover, there is some evidence that suggests that OXR agonists could be useful in controlling pain and other conditions resulting from excessive stress while OXR antagonists could help during drug addiction withdrawal.[5,6] Taken together, these findings suggest that OX2R may be a potentially important therapeutic target for treatment of sleep disorders, obesity, emotional stress, and addiction.[7]

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 OX2R Reporter Assay Kit consists of a 96-well plate coated with a transfection complex containing DNA constructs for OX2R, a recombinant G-protein, and a cAMP response element regulated Secreted Alkaline Phosphatase (SEAP) reporter (OX2R Reverse Transfection Strip Plate). Cells grown on the transfection complex will express OX2R at the cell surface and the recombinant G-protein inside the cell. Binding of agonists to OX2R initiates a signal transduction cascade resulting in expression of SEAP which is secreted into the cell culture medium. Aliquots of culture medium are removed at time intervals beginning at about 16 hours and SEAP activity is measured following addition of a luminescence-based alkaline phosphatase substrate provided in the kit. The kit is easy to use and can be easily adapted to high throughput screening for therapeutic compounds regulating activation of OX2R. A known OX2R agonist, Orexin A, is included in the kit for use as a positive control. The kit provides sufficient reagent to measure SEAP activity at three time points, using the black plates provided.

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 Orexin A 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 Orexin A to run a control dose-response curve as well. Record the contents of each well on the template sheet provided on page 14.

Addition of Cells to the Reverse Transfection Plate

IMPORTANT

Before starting the experiment, dilute Penicillin-Streptomycin (100X, Gibco 15140-122) 1:100 in culture medium used for your cells. This will be the culture medium for your experiment.

1. Remove the Orexin 2 Receptor Reverse Transfection Strip Plate (Item No. 600251) from the freezer and allow 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 50,000-100,000 cells/well in 200 µl of culture medium containing 10% FBS. Place the plate in a 37°C incubator and incubate overnight or up to 24 hours.

Cell Stimulation

1. After 16-24 hours of incubation, aspirate the culture media from each well.
2. Add 100 µl of culture media containing 0.5% FBS to each well.
3. Prepare test compounds at 2X the desired final concentration in serum-free media and pipette 100 µl to the assigned wells. Wells containing untreated cells receive 100 µl of serum-free media only. For positive controls using the provided Orexin A, dilute the Orexin A Positive Control (Item No. 600242) 1:1,000 in the serum-free culture media and add 100 µl to corresponding wells. At this concentration, Orexin A induces a 5-8 fold increase in SEAP activity, depending on the cell type and stimulation time used. The stock solution should be stored at -80°C.

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 refrigerator and allow to equilibrate to room temperature. *NOTE: Vortex the substrate vigorously prior to use*

1. After 16-30 hours of stimulation with test compounds, transfer the plate from the incubator to a culture hood.
2. In the culture hood, remove 10 µl of culture media from each well to a corresponding well of a 96-Well Solid Plate (black) with lid (Item No. 10011297). Cover the plate with the lid.
3. 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.
4. Remove the plate from the 65°C incubator and allow to equilibrate to room temperature.
5. Add 50 µl of substrate to each well, shake briefly, and incubate the plate at room temperature for 20-30 minutes.
6. Read the plate with a plate reader capable of detecting a chemiluminescence signal.

NOTE: The plate should be read immediately after 20-30 minutes of 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 11, for Orexin A curve.

For each compound, normalize the 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).

NOTE: This kit could be used to characterize antagonists by co-incubation of the experimental compound with a fixed dose of Orexin A such as 3-10 nM.

Performance Characteristics

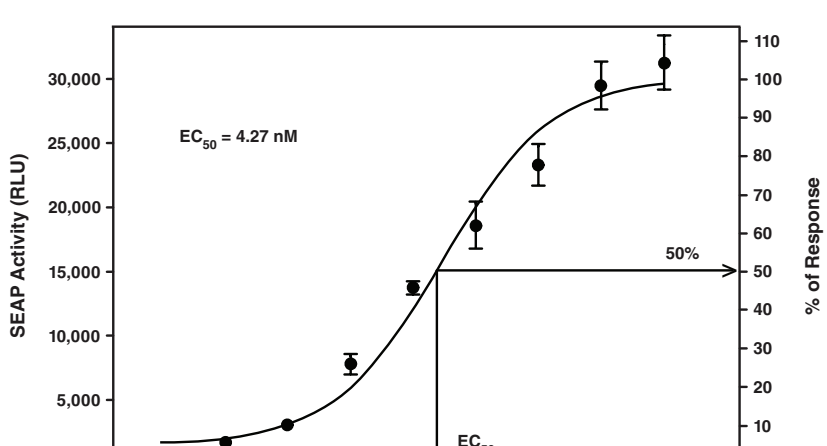

Figure 1. SEAP activity in HEK293 cells transiently-transfected with OX2R in response to Orexin A stimulation. HEK293 cells were plated in a OX2R Reverse Transfection Strip Plate at a density of 60,000 cells/well and incubated overnight. The next day, cells were treated with different doses of Orexin A as indicated above. After 24 hours of stimulation, 10 µl of culture media was removed from each well and the SEAP activity from each sample was measured according to the protocol described on page 9. The calculated EC_{50} value from the fitted curve is 4.27 nM.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	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	Plate cells more sparsely
No signal	A. Contamination B. Layer lost	A. Keep plate in sterile environment B. Gently aspirate medium; do not disturb cell layer

References

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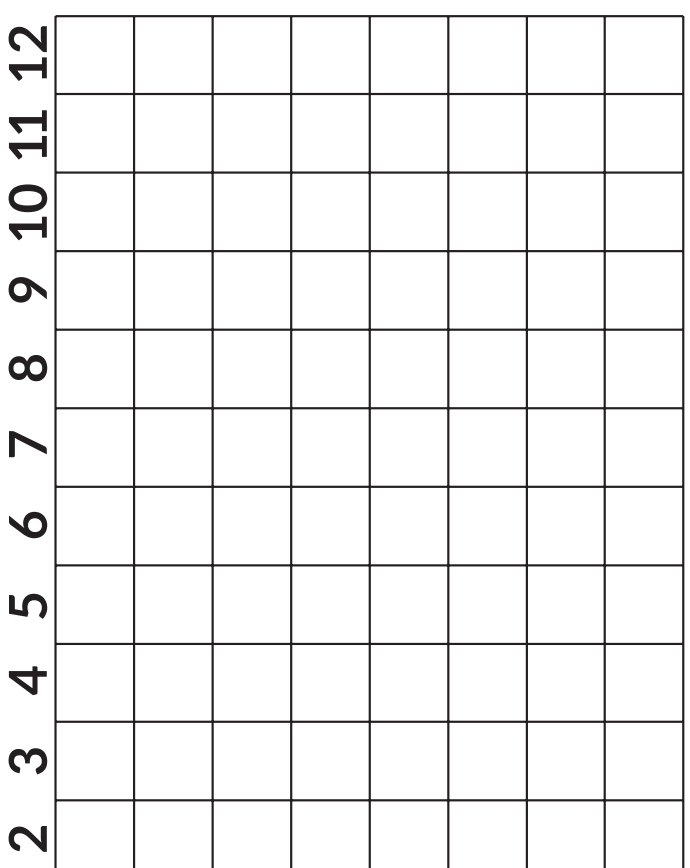
3. Thannickal, T.C., Moore, R.Y., Nienhuis, R., *et al.* Reduced number of hypocretin neurons in human narcolepsy. *Neuron* **27**, 469-474 (2000).
4. Rodgers, R.J., Ishii, Y., Halford, J.C.G., *et al.* Orexins and appetite regulation. *Neuropeptides* **36(5)**, 303-325 (2002).
5. Xie, X., Wisor, J.P., Hara, J., *et al.* Hypocretin/orexin and nociceptin/orphanin FQ coordinately regulate analgesia in a mouse model of stress-induced analgesia. *J. Clin. Invest.* **118(7)**, 2471-2481 (2008).
6. Harris, G.C., Wimmer, M., and Aston-Jones, G. A role for lateral hypothalamic orexin neurons in reward seeking. *Nature* **437**, 556-559 (2005).
7. Tsujino, N. and Sakurai, T. Orexin/hypocretin: A neuropeptide at the interface of sleep, energy homeostasis, and reward system. *Pharmacol. Rev.* **61**, 162-176 (2009).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.