Calcitonin Receptor (human) Reporter Assay Kit

Item No. 502903

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
401182	CTR Reverse Transfection Strip Plate	1 plate	-20°C
401183	KBP-066 Positive Control (10 µM)	1 vial/50 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 ea	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.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Calcitonin Receptor (human) Reporter Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

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

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. HEK293T or HEK293T/17 cells; both cell lines can be obtained from ATCC
2. Dulbecco's modified Eagle's medium (DMEM)
3. Fetal bovine serum (FBS)
4. Penicillin-streptomycin (100X) (ThermoFisher™ Scientific, Catalog No. 15140-122)
5. A plate reader capable of measuring luminescence
6. Adjustable pipettes and a multichannel pipettor
7. An incubator set at 65°C

INTRODUCTION

Background

Calcitonin receptor (CTR) is a class B1 G protein-coupled receptor (GPCR) and member of the secretin or 'B' family.[1,2] It is expressed in brain, kidney, lung, gastrointestinal tract, and reproductive organs and in leukocytes and osteoclasts.[2] CTR is activated by the endogenous peptide hormone calcitonin.[2,3] CTR activation in osteoclasts inhibits bone resorption and has been clinically exploited in the treatment of various bone diseases, including osteoporosis, Paget's disease, and hypercalcemia of malignancy. Loss-of-function mutations in *CALCR*, the gene encoding CTR, are associated with poor prognosis in glioblastoma.[4] In addition to its role as a standalone receptor, CTR can interact with the receptor activity-modifying protein (RAMP) family to form distinct amylin and CGRP receptors, which have roles in the regulation of appetite, satiety, glucose metabolism, and migraine-related pain and inflammation.[2,5,6]

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 has been 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 co-transfection efficiency for multiple plasmids.

Cayman's Calcitonin Receptor (human) Reporter Assay Kit consists of a 96-well plate coated with a transfection complex containing DNA constructs for expressing CTR and a cAMP response element-regulated secreted alkaline phosphatase (SEAP) reporter (CTR Reverse Transfection Strip Plate). Cells grown on the transfection complex will express CTR at the cell surface within 24 hours. Binding of agonists to CTR initiates a signal transduction cascade through the Gαs and adenylate cyclase pathway resulting in the expression of SEAP, which is secreted into the cell culture medium. Aliquots of culture medium are collected at 6-8 hours after stimulation and SEAP activity is measured following the addition of a luminescence-based alkaline phosphatase substrate provided in the kit (SEAP Substrate). The kit is easy to use and can be readily applied to high-throughput screening for therapeutic compounds regulating the activation of CTR. A synthetic peptide agonist, KBP-066, is included for use as a positive control. The kit provides a sufficient volume of reagents to measure SEAP activity at three time points using the three included white assay plates.

Principle Of This Assay

The binding of an agonist to CTR activates $G\alpha_s$ and triggers the cAMP/PKA/CREB signaling pathway. Phosphorylated CREB binds to the CREB-responsive element (CRE) in the promoter region of the reporter and induces the expression of SEAP. This secreted alkaline phosphatase is more stable than endogenous alkaline phosphatase, and the activity of SEAP is measured as a luminescence signal produced after incubation with the provided substrate.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical experiment will include wells containing cells treated with KBP-066 as a positive control, treated with experimental compounds, or left untreated. It is recommended that each treatment be performed at least in triplicate. To determine the EC_{50} value of a compound, serial dilutions of the compound should be included in the assay. The volume of KBP-066 Positive Control (10 µM) is sufficient to run a full dose-response curve with replicates or to serve as a control at approximately EC_{80} for testing inhibitors on the whole plate. Record the contents of each well on the template sheet provided (see page 17).

Addition of Cells to the Reverse Transfection Plate

IMPORTANT

Before starting the experiment, pre-warm the required volume of culture medium and make sure a sufficient number of actively growing cells are available.

1. Remove the CTR Reverse Transfection Strip Plate (Item No. 401182) from the freezer and allow it to equilibrate to room temperature inside the sealed bag. After it has reached room temperature, clean the bag with 70% alcohol before opening it and place the plate inside a cell culture hood.

   NOTE: If not using the whole plate within one experiment, remove the number of strips needed, place the remaining strips back in the bag, and store them in a desiccator protected from UV light at room temperature for up to a week. Alternatively, the remaining strips can be sealed in the bag with the desiccant pack and stored at -20°C for up to two months.

2. Seed HEK293T or HEK293T/17 cells at a density of 50,000-60,000 cells/well in 200 µl of DMEM containing 10% FBS and 1X penicillin/streptomycin and keep it in the hood for 30-45 minutes.
3. Place the plate in a 37°C CO_2 incubator and incubate for 16-20 hours.

Cell Stimulation

1. After 16-20 hours of incubation, carefully aspirate the culture medium from each well.
2. Replenish the cells with 150 µl of pre-warmed stimulation medium (DMEM with 0.5% FBS) per well.
3. Prepare test compounds at 4X the desired final concentration in stimulation medium and pipette 50 µl into the assigned wells. Pipette 50 µl of stimulation medium into the wells designated for untreated cells. For positive control wells, dilute the KBP-066 Positive Control (10 µM) (Item No. 401183) 1:1,000 in stimulation medium and add 50 µl to the positive control wells. At this concentration (2.5 nM), KBP-066 will induce a >5-fold increase in SEAP activity in 6-8 hours compared to the untreated control.

Performing the SEAP Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Avoid introducing bubbles to the well.
- 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 it to equilibrate to room temperature.

1. After 6-8 hours of stimulation with test compounds and controls, transfer the plate from the incubator to a cell culture hood.
2. Inside the culture hood, use a multichannel pipette to gently pipette up and down a few times. Collect 10 µl of medium from each well and transfer it to the corresponding well of a 96-Well Solid Plate (white) (Item No. 700029).

   NOTE: Avoid contact of the pipette tip with the plate bottom to minimize disruption of the cell layer. Perform this step inside the cell culture hood and return the plate to the incubator if sampling at later time point(s) is needed.
3. Cover the assay plate with the 96-Well Cover Sheet (Item No. 400012) and incubate the plate in a 65°C incubator for 30 minutes to heat inactivate endogenous alkaline phosphatase. The SEAP expressed in this assay is stable under this condition.
4. Remove the plate from the 65°C incubator, remove the cover sheet, and allow the plate to cool to room temperature.
5. Add 50 µl SEAP Substrate to each well, shake/tap briefly to mix, and incubate the plate at room temperature for 5-15 minutes.
6. Scan the plate for luminescence in a microplate reader.

NOTE: The plate should be read immediately after 5-15 minutes of incubation with SEAP Substrate. When multiple plates are processed at the same time, the time interval between plates for the addition of substrate and plate reading should be consistent.

ANALYSIS

Calculations

Determination of EC_{50}

The term half-maximal effective concentration (EC_{50}) refers to the concentration of a drug that induces a response halfway between the baseline and maximum after a 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 KBP-066 dose-response curve.

For each compound, normalize 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 the percent response *versus* log drug concentration. In the resulting sigmoidal 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

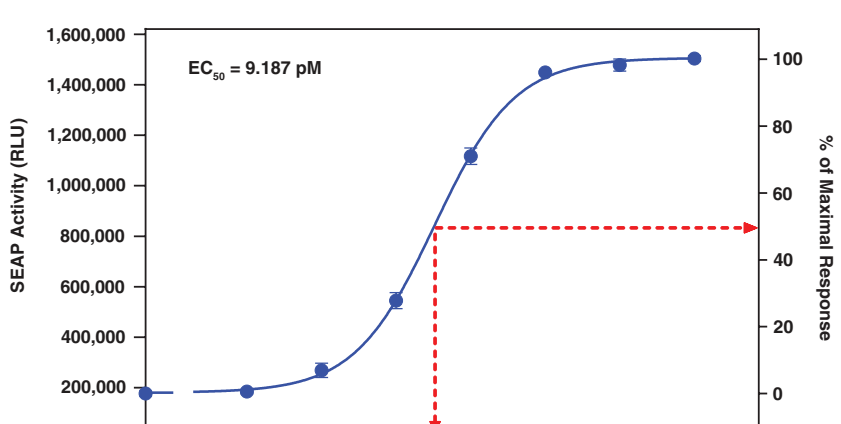


Figure 1. SEAP activity in HEK293T cells transiently transfected with CTR in response to KBP-066 stimulation. HEK293T cells were seeded on an CTR Reverse Transfection Strip Plate at a density of 50,000 cells/well and incubated overnight. The next day, the cells were treated with different doses of KBP-066 up to 2.5 nM in stimulation medium. After six hours of stimulation, 10 µl of culture medium was collected from each well and the SEAP activity of each sample was measured according to the **Performing the SEAP Assay** section (see page 10). The calculated EC_{50} value for KBP-066 from the fitted curve is 9.19 pM, and the Z' value of the assay is >0.9.

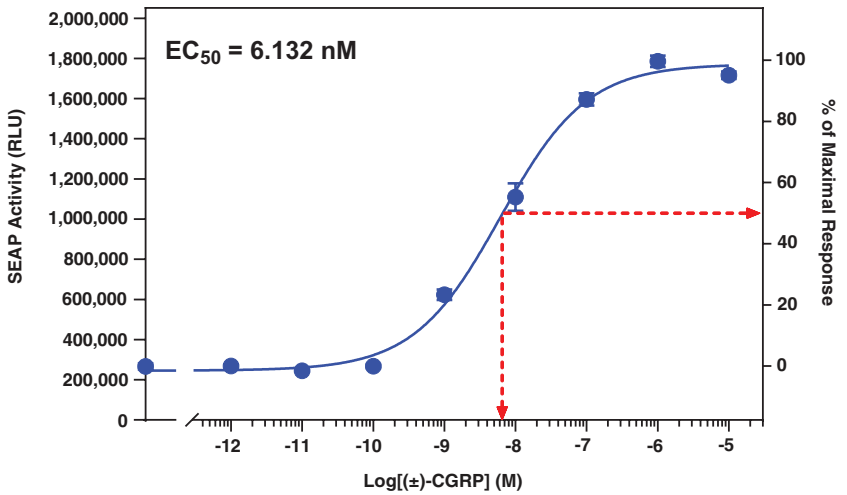


Figure 2. Dose-response curve of α-CGRP in the CTR reporter assay. Using the same protocol, another agonist for the calcitonin receptor, α-CGRP, was examined in the reporter assay in a separate experiment. The cells were treated with serial dilutions of α-CGRP up to 10 µM in stimulation medium. After six hours of stimulation, 10 µl of culture medium was collected for measuring SEAP activity. The calculated EC_{50} value for α-CGRP from the fitted curve is 6.13 nM.

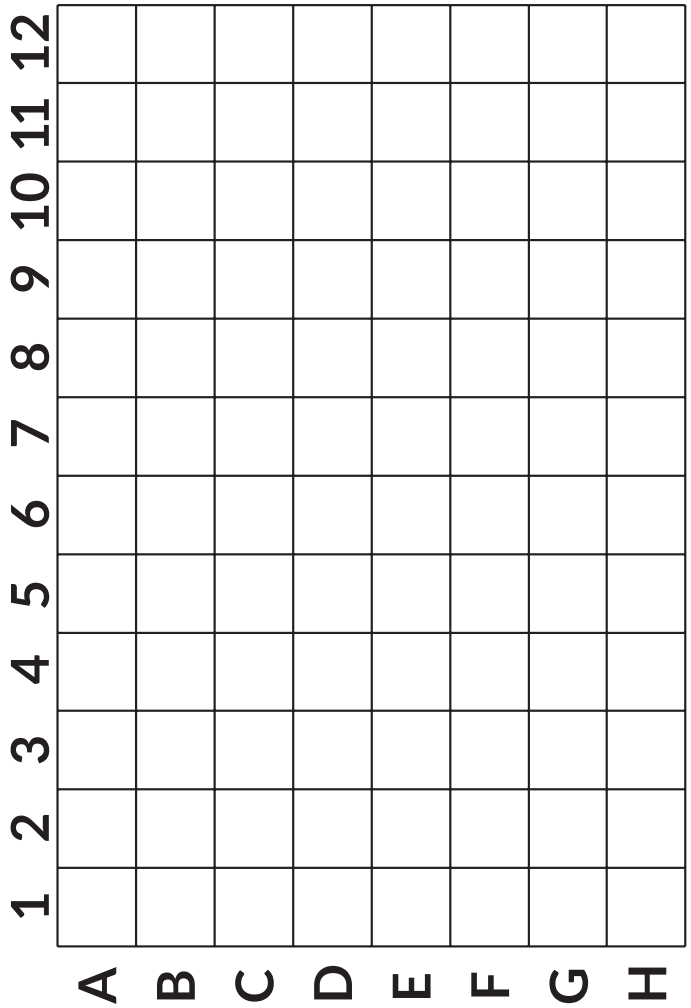
NOTE: The fold of stimulation, Z' value, and calculated EC_{50} may vary with cell lines, cell passage, and culture conditions.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Dispersion of replicates or erratic response curve of test compounds	A. Uneven cell distribution B. Poor pipetting C. Not well mixed at the sampling step D. Bubble in assay wells	A. Make sure the cells are in a homogenous suspension at the time of plating and allow the cells to sit for 30-45 minutes before placing into the incubator B. Pipette carefully C. Pipette up and down a few times before collecting a sample D. Carefully tap the side of the plate to remove bubbles
Low reading in wells	A. Reading time was too short B. Samples overheated/ dried C. The SEAP substrate was too cold	A. Increase the integration time B. Do not heat-inactivate the sample plate above 65°C or longer than 30 minutes C. Warm up the SEAP substrate to RT before use
Sample signal is too strong	A. Cell density was too high B. Insufficient heat inactivation of endogenous alkaline phosphatase activity	A. Reduce cell plating density B. Correct the duration or temperature of heat inactivation step
Poor control curve/ signal	A. Control compound degraded B. Pipetting error C. Splashing of sample D. Volume carry-over during dilution	A. Avoid freeze-thaw of positive control B. Check pipette volume C. Dispense carefully D. Use new tip for each pipetting

References

1. Barwell, J., Gingell, J.J., Watkins, H.A., *et al.* Calcitonin and calcitonin receptor-like receptors: Common themes with family B GPCRs? *Brit. J. Pharmacol.* **166**, 51-65 (2012)
2. dal Maso, E., Glukhova, A., Zhu, Y., *et al.* The molecular control of calcitonin receptor signaling. *ACS Pharmacol. Transl. Sci.* **2**, 31-51 (2019).
3. Masi, L. and Brandi, M.L. Calcitonin and calcitonin receptors. *Clin. Cases Miner. Bone Metab.* **4(2)**, 117-122 (2007).
4. Pal, J., Patil, V., Kumar, A., *et al.* Loss-of-function mutations in calcitonin receptor (*CALCR*) identify highly aggressive glioblastoma with poor outcome. *Clin. Cancer Res.* **24(6)**, 1448-1458 (2018).
5. Bower, R.L., Yule, L., Rees, T.A., *et al.* Molecular signature for receptor engagement in the metabolic peptide hormone amylin. *ACS Pharmacol. Transl. Sci.* **1**, 32-49 (2018).
6. Secher, A., Lutz, T.A., and Raun, K. The story of amylin: From physiology to therapy. *Nat. Metab.* **8(2)**, 299-312 (2026).



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.