FFAR1 (GPR40) Reporter Assay Kit

Item No. 601190

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GENERAL INFORMATION

Materials Supplied

This kit will arrive packaged as a -20°C kit. After opening the kit, store individual components as stated below.

Item Number	Item	100 Tests Quantity/Size	Storage
601191	FFAR1 Reverse Transfection Strip Plate	1 plate	-20°C
601192	FFAR1 Assay GW 9508 Positive Control (10 mM)	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

This kit will perform as specified if stored as directed and used before the expiration date indicated on the outside of the box. Upon arrival of the kit, store each component at appropriate temperature accordingly, see page 3.

Materials Needed But Not Supplied

1. HEK293T cells, available from ATCC
2. Dulbecco's Modified Eagle's Medium (DMEM)
3. Fetal bovine serum (FBS)
4. Penicillin-streptomycin (100X)
5. Opti-MEM I reduced Reduced Serum Medium (ThermoFisher 31985062)
6. A plate reader capable of measuring luminescence
7. Adjustable and multichannel pipettes with pipette tips
8. An incubator/oven set at 65°C

INTRODUCTION

Background

Free fatty acids (FFAs) are energy-generating essential nutrients that also act as signaling molecules in the regulation of various cellular functions. They are derived from food sources and from lipase metabolized triglyceride stores in the body. Several previously orphan G protein-coupled receptors (GPCRs) have been identified to be FFA receptors (FFARs). This family of receptors can be subdivided by their ligand profiles based on the length of carbon chains in the FFA.

FFAR1/GPR40 is a GPCR for long-chain fatty acids such as the ω-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid. It is involved in the regulation of insulin release from pancreatic β cells and mediates triglyceride-induced secretion of incretins GLP-1 and GIP from intestinal endocrine cells.[1-3] It has been clinically proven to be an anti-diabetes drug target, which enhances insulin secretion in patients with type 2 diabetes.[4]

Binding of ligand to FFAR1 triggers an activation signal transmitted primarily through the $G\alpha_{q/11}$ pathway, although in some cases $G\alpha_s$ is also involved.[5] The activated $G\alpha_{q/11}$-protein complex leads to the activation of phospholipase C (PLC) which hydrolyses phosphoinositol-containing lipids to generate 1,4,5-trisphosphate (IP_3) and diacylglycerol. The IP_3 produced in turn mediates the intracellular release of calcium from internal storage. Therefore, the activation of FFAR1 is typically measured by calcium mobilization.

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 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 FFAR1 (GPR40) Reporter Assay Kit consists of a 96-well plate coated with a transfection complex containing DNA constructs for FFAR1, an engineered G protein that directs $G\alpha_q$ activation signals to the $G\alpha_s$ pathway, and a cAMP response element-regulated secreted alkaline phosphatase (SEAP) reporter (FFAR1 reverse transfection strip plate). Cells grown on the transfection complex will express FFAR1 at the cell surface and the recombinant G protein inside the cell. Binding of agonists to FFAR1 initiates a signal transduction cascade resulting in expression of SEAP which is secreted into the cell culture medium. Aliquots of culture medium are collected 16-24 hours after stimulation 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 readily applied to high-throughput screening for therapeutic compounds regulating activation of FFAR1. A known FFAR1 agonist, GW 9508, 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 three included white assay plates.

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 GW 9508 provided in the kit (positive control), wells with cells treated with experimental compounds, and wells of untreated cells. It is recommended that each treatment be performed at least in triplicate. In order to determine the EC_{50} value of a test compound, serial dilutions of the compound should be included in the assay. The positive control GW 9508 provided is sufficient to run a full dose-response curve with replicates up to 10 µM. 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 the penicillin-streptomycin (100X) 1:100 in the culture medium used for your cells. This will be the culture medium for your experiment.

1. Remove the unopened FFAR1 reporter reverse transfection strip plate (Item No. 601191) from the freezer and allow to equilibrate to room temperature. After it has reached room temperature, spray the exterior of the bag with 70% alcohol before opening the bag. Place the bag in the hood and remove the plate 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 one week. Alternatively, the bag can be vacuum-sealed and stored at -20°C for up to two months.*

2. Seed HEK293T cells at a density of 40,000-60,000 cells/well in 200 µl of culture medium (DMEM plus 10% FBS and 1X penicillin/streptomycin). Let the plate sit inside the hood for 30-45 minutes.
3. Place the plate in a 37°C incubator and incubate for 18-24 hours.

Cell Stimulation

1. After 18-24 hours of incubation, aspirate the culture medium from each well carefully.
2. Replenish the cell with 100 µl pre-warmed stimulation medium (serum-free Opti-MEM) per well.
3. Prepare test compounds at 2X the desired final concentration in serum-free Opti-MEM and pipette 100 µl to the assigned wells. Wells containing untreated cells receive 100 µl of Opti-MEM. For positive controls using the provided GW 9508, dilute the 10 mM GW 9508 positive control (Item No. 601192) 1:1,000 in the Opti-MEM and add 100 µl to positive control wells. At this concentration (5 µM), GW 9508 induces a >3-fold increase in SEAP activity in 16-24 hours over 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 to equilibrate to room temperature.

1. After 16-24 hours of stimulation with test compounds and controls, transfer the plate from the incubator to a tissue culture hood.
2. Inside the culture hood, use a multichannel pipette to gently pipette the medium up and down a few times and transfer 10 µl from each well onto corresponding well of a white 96-well assay plate (Item No. 700029). Return the culture plate to the incubator if planning to sample at a later time.
3. Seal the assay plate with the provided plate cover (Item No. 400012) and incubate the plate in an oven set at 65°C for 30 minutes to heat-inactivate endogenous (non-SEAP) alkaline phosphatase. The SEAP expressed in this assay is stable under this condition.
4. Remove the plate from the 65°C incubator, remove the plate cover, and allow the plate to cool down to room temperature.
5. Add 50 µl substrate to each well, shake 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. When multiple plates are processed at the same time, the time interval between plates for addition of substrate and for plate reading should be consistent.

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 a typical GW 9508 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:

% Response at X Concentration =

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

Graph the % response *versus* the 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

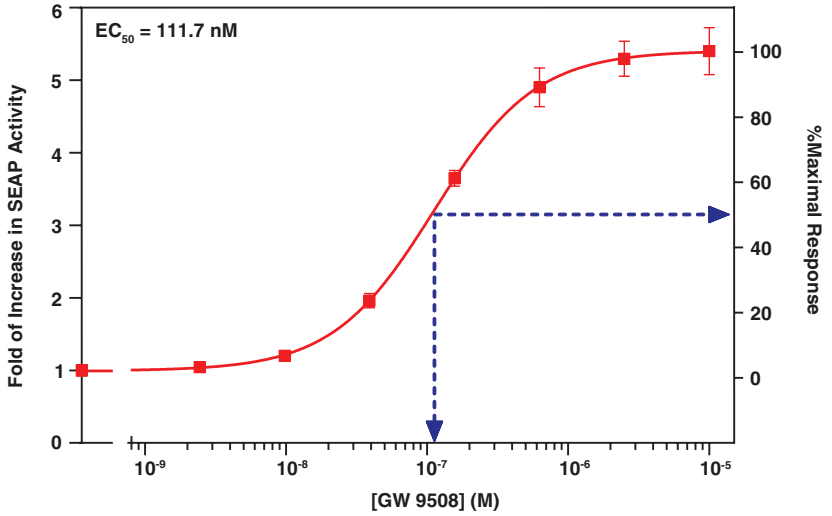


Figure 1. SEAP activity response to stimulation with GW 9508 in HEK293T cells transiently transfected with FFAR1 receptor. HEK293T cells were plated on a FFAR1 reverse transfection strip plate at a density of 50,000 cells/well and incubated overnight. The next day, cells were treated in triplicate with different doses of GW 9508 in serum-free medium in triplicates. After 24 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 protocol described on page 9. The calculated EC_{50} value from the fitted curve is 111.7 nM and the Z' value is >0.7.

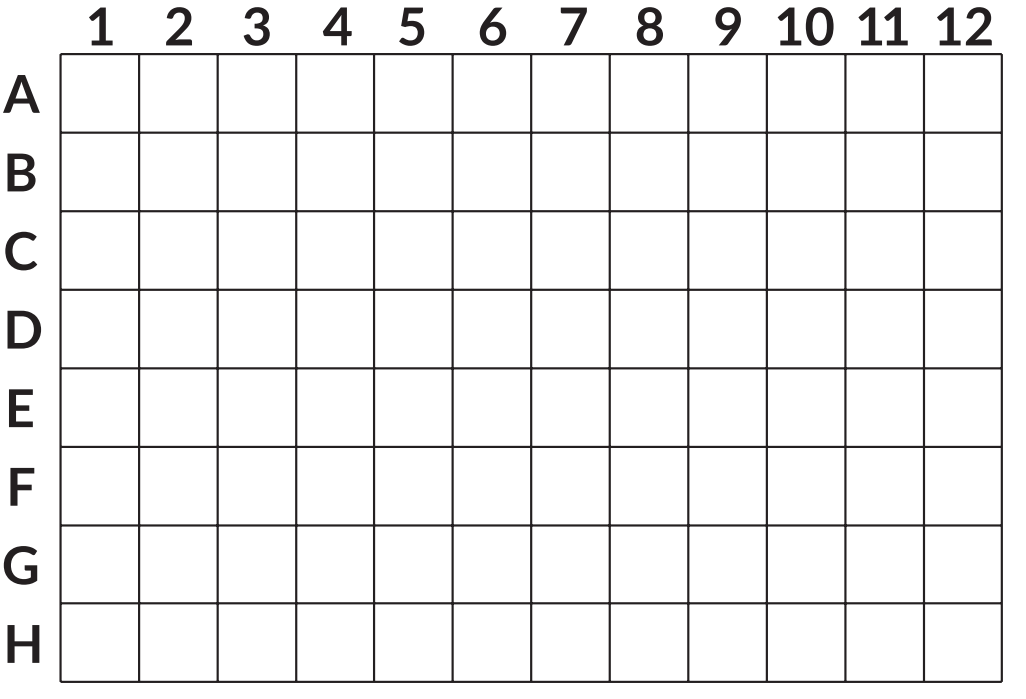
NOTE: The fold stimulation, Z' value, and calculated EC_{50} may vary with cell batches and culture conditions.

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 when sampling D. Bubble in assay wells	A. Make sure cells are in homogenous suspension at plating and allow the cells to sit for 30-45 min before putting into incubator B. Pipette carefully C. Pipette up and down a few times before collecting sample D. Carefully tap the side of the plate to remove bubbles
Low reading in wells	A. Reading time is too short B. Samples overheated/dried C. The substrate is too cold	A. Increase the integration time B. Keep the plate away from heat source C. Warm-up the substrate to room temperature 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 free-thaw of positive control B. Check pipette volume C. Dispense carefully D. Use new tip for each pipetting

References

1. Burant, C.F. Activation of GPR40 as a therapeutic target for the treatment of type 2 diabetes. *Diabetes Care* **36 (Suppl 2)**, S175-S179 (2013).
2. Tanaka, H., Yoshida, S., Oshima, H., *et al.* Chronic treatment with novel GPR40 agonists improve whole-body glucose metabolism based on the glucose-dependent insulin secretion. *J. Pharmacol. Exp. Ther.* **346(3)**, 443-452 (2013).
3. Itoh, Y., Kawamata, Y., Harada, M., *et al.* Free fatty acids regulate insulin secretion from pancreatic β cells through GPR40. *Nature* **422**, 173-176 (2003).
4. Negoro, N., Sasaki, S., Mikami, S., *et al.* Discovery of TAK-875: A potent, selective, and orally bioavailable GPR40 agonist. *ACS Med. Chem. Lett.* **1(6)**, 290-294 (2010).
5. Mancini, A.D. and Poitout, V. The fatty acid receptor FFA1/GPR40 a decade later: How much do we know? *Trends Endocrinol. Metab.* **24(8)**, 398-407 (2013).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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.

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