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## TGR5 (GP-BAR1) Reporter Assay Kit

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Item No. 601440

[www.caymanchem.com](http://www.caymanchem.com)

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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
601441	TGR5 Reverse Transfection Strip Plate	1 plate	-20°C
601442	TGR5 Receptor Agonist 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

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

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. HEK293 or HEK293T cells, available from ATCC
2. Culture medium used for maintenance of the cells (DMEM with 10% FBS and 1X penicillin-streptomycin) (100X Penicillin-Streptomycin from Thermo Fisher)
3. Stimulation medium (DMEM + 1X penicillin-streptomycin)
4. A plate reader capable of measuring luminescence
5. Adjustable and multichannel pipettes with pipette tips
6. An incubator/oven set at 65°C

## Background

Bile acids are a diverse class of amphipathic molecules originally formed in liver and modified by microorganisms in the intestine. In addition to their conventional role in dietary lipid absorption, bile acids have been recognized as signaling molecules that regulate the synthesis and metabolism of bile acids, glucose homeostasis, and energy expenditure. Bile acid receptors are, therefore, considered drug targets for various metabolic diseases including obesity and type 2 diabetes mellitus. Several nuclear receptors including farnesoid X receptor (FXR), pregnane X receptor (PXR), vitamin D<sub>3</sub> receptor (VDR), and constitutive androstane receptor (CAR), have been found to interact with certain bile acids and carry out some of their signaling pathways and processes.<sup>1</sup> The G protein-coupled membrane receptor for bile acid, TGR5 or GP-BAR1, was identified in 2002.<sup>2</sup> Since then, it has been an interesting drug target in metabolism and inflammation and it is the most extensively studied bile acid receptor.<sup>3,4</sup>

TGR5 is a Gα<sub>s</sub>-coupled receptor expressed in various tissues and cell types such as macrophages, enteroendocrine cells, brown adipocytes, skeletal muscles, enteric neurons, biliary epithelial cells, etc.<sup>4,5</sup> Taurine- or glycine-conjugated and unconjugated bile acids are endogenous agonists for TGR5, while lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), and cholic acid (CA) are the most potent activators in the order of decreasing potency.<sup>2</sup> Activation of TGR5 by agonists leads to increase in intracellular cAMP, which signals the reduction of inflammatory cytokine expression in macrophage, increase of glucagon-like peptide (GLP-1) secretion in intestine, and energy expenditure in brown adipose tissue and muscle.<sup>1</sup>

## 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 immobilized 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 TGR5 (GP-BAR1) Reporter Assay Kit consists of a 96-well plate coated with a transfection complex containing DNA constructs for expressing TGR5 and a cAMP response element regulated secreted alkaline phosphatase (SEAP) reporter (TGR5 reverse transfection strip plate). Cells grown on the transfection complex will express TGR5 at the cell surface within 24 hours. Binding of agonists to TGR5 initiates a signal transduction cascade through the Gα<sub>s</sub> 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. The kit is easy to use and can be readily applied to high-throughput screening for therapeutic compounds regulating activation of TGR5. A selective synthetic agonist, the TGR5 Receptor Agonist (3-[2-chlorophenyl]-N-[4-chlorophenyl]-N,5-dimethyl-4-isoxazolecarboxamide), is included in the kit for use as a positive control.<sup>6</sup> The kit provides sufficient reagent to measure SEAP activity at three time points using the three included white assay plates.

## Plate Set Up

There is no specific pattern for using the wells on the plate. A typical experiment will include: wells with cells treated with TGR5 receptor agonist 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 TGR5 Receptor Agonist Positive Control provided is sufficient to run a full dose-response curve with replicates up to 10  $\mu$ M. 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, pre-warm enough volume of culture medium and make sure sufficient actively growing cells would be available.

1. Remove the TGR5 Reverse Transfection Strip Plate (Item No. 601441) from the freezer and allow to equilibrate to room temperature within sealed bag.
2. After plate has reached room temperature, clean the bag with 70% alcohol before opening the bag and put the plate inside the hood.
3. Seed HEK293 or HEK293T cells at a density of 30,000-50,000 cells/well in 200  $\mu$ l of complete culture medium.
4. Allow the plate to sit inside the hood for 30-45 minutes.
5. Place the plate in a 37°C CO<sub>2</sub> incubator and incubate for 18-24 hours.

*NOTE: If the whole plate would not be completely used within one 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, remaining strips can be sealed in the bag with desiccant pack and stored at -20°C for up to two months.*

## Cell Stimulation

1. After 18-24 hours of incubation, aspirate the culture medium from each well carefully.
2. Replenish the cell with 150  $\mu$ l pre-warmed stimulation medium per well.
3. Prepare test compounds at 4X the desired final concentration in serum-free stimulation medium and pipette 50  $\mu$ l to the assigned wells.
4. Untreated cells receive 50  $\mu$ l of stimulation medium per well.
5. For positive control wells, dilute the provided TGR5 Receptor Agonist Positive Control (10 mM) (Item No. 601442) 1:250 in the stimulation medium and add 50  $\mu$ l per well.

*NOTE: At 10  $\mu$ M, the TGR5 Receptor Agonist Positive Control typically induces a >5-fold increase in SEAP activity in 6-8 hours over untreated control. Prepare aliquots of TGR5 Receptor Agonist Positive Control if needed to minimize freeze-thaw cycles.*

## 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 6-8 hours of stimulation with test compounds and controls, use a multichannel pipette to gently pipette up and down a few times and collect 10  $\mu$ l media from each well onto corresponding well of a 96-Well Solid Plate (white) (Item No. 700029).

*NOTE: Avoid contact of pipette tip with plate bottom to minimize disruption of cell layer. Perform inside cell culture hood and return the plate into incubator if sampling at later time point(s) is needed.*

2. Cover the assay plate with provided 96-Well Cover Sheet (Item No. 400012).  
*NOTE: Sealed sample plate may be stored at -20°C if not assaying immediately.*
3. Incubate the plate in an oven set at 65°C for 30 minutes to heat inactivate endogenous alkaline phosphatase.
4. Remove the plate from the 65°C incubator, discard the cover sheet, and allow the plate to cool down to room temperature.
5. Add 50  $\mu$ l 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 addition of substrate and for plate reading should be consistent.*

## Calculations

### Determination of EC<sub>50</sub>

The term half maximal effective concentration (EC<sub>50</sub>) 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 TGR5 Receptor Agonist Positive Control dose-response curve.

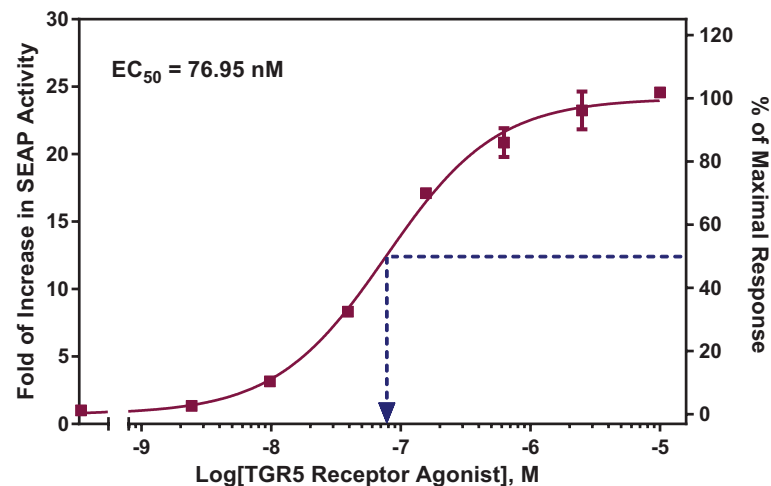
For each compound, normalize the Relative Luminescent Unit (RLU) results to run from 0% (no drug added) to 100% (saturating dose) by using the following formula:

% Response at X Concentration =

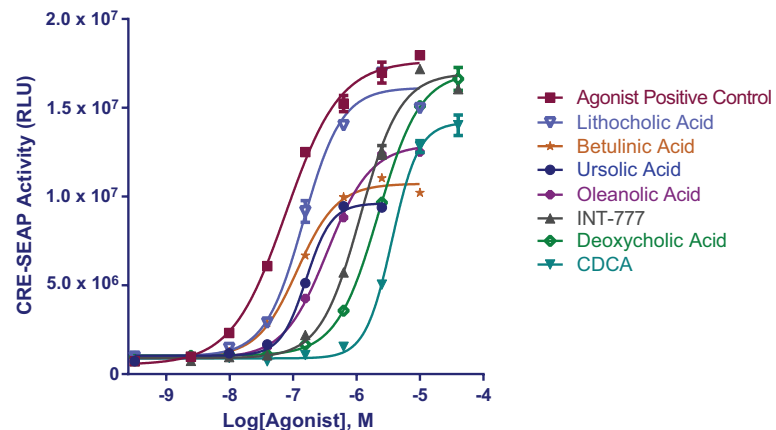
$$\left[ \frac{(\text{RLU at X concentration}) - (\text{RLU of untreated cells})}{\text{Maximal RLU (saturation)} - (\text{RLU of untreated cells})} \right] \times 100$$

Graph the % response *versus* the log drug concentration. In the resulting sigmoidal dose-response curve, find the best-fit value for the log EC<sub>50</sub> (the concentration that gives a 50% response; the middle of the curve).

## Performance Characteristics



**Figure 1.** SEAP activity in HEK293T cells transiently transfected with TGR5 receptor in response to TGR5 Receptor Agonist Positive Control stimulation. HEK293T cells were plated in a TGR5 Reverse Transfection Strip Plate at a density of 40,000 cells/well and incubated overnight. The next day, cells were treated with different doses of TGR5 Receptor Agonist Positive Control up to 10  $\mu$ M in serum-free stimulation medium. After seven hours of stimulation, 10  $\mu$ l of culture media was collected from each well and the SEAP activity of each sample was measured according to the protocol described on page 11. The calculated EC<sub>50</sub> value from the fitted curve is 76.95 nM and the Z' value is >0.8. *NOTE: The fold of stimulation, Z' value, and calculated EC<sub>50</sub> may vary with cell lines, cell passages, and culture conditions.*



**Figure 2. Validation of TGR5 reporter assay with different classes of agonists.** In addition to the TGR5 Receptor Agonist Positive Control, seven known TGR5 receptor agonists under different classifications (including common bile acids, natural bio-product, synthetic, steroidal, non-steroidal, full agonists, and partial agonists)<sup>4</sup> were examined in the TGR5 (GP-BAR1) Reporter Assay. HEK293T cells transfected on two strip well plates were treated with serial dilutions of TGR5 Receptor Agonist Positive Control, LCA, betulinic acid, ursolic acid, oleanolic acid, INT-777, DCA, and CDCA in triplicated wells. Media samples were collected and analyzed as described above. The relative potencies of bile acids LCA, DCA, and CDCA are consistent with literature.<sup>1,2</sup> Betulinic acid and oleanolic acid are natural non-steroidal partial agonists of TGR5 that both exhibited lower maximal responses than the full agonists as expected. In addition, other known agonists including INT-777 and ursolic acid also demonstrated dose-dependent responses in the assay.

## RESOURCES

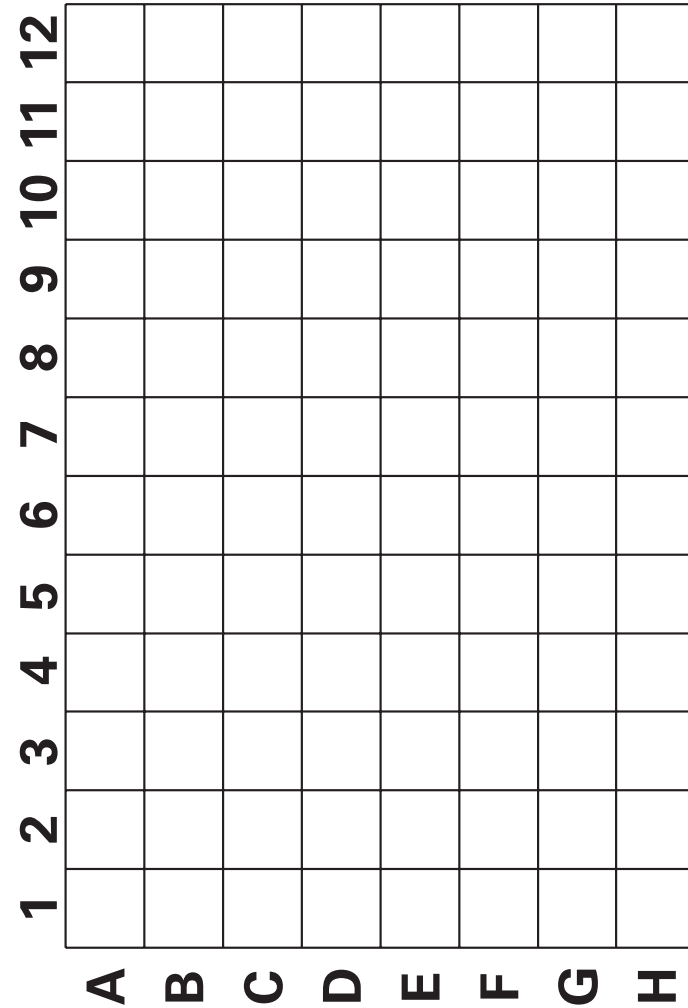
### Troubleshooting

Problem	Possible Causes	Recommended Solutions
Dispersion of replicates or erratic response curve of test compounds	<ul style="list-style-type: none"> <li>A. Uneven cell distribution</li> <li>B. Poor pipetting</li> <li>C. Not well mixed when sampling</li> <li>D. Bubble in assay wells</li> </ul>	<ul style="list-style-type: none"> <li>A. Make sure cells are in homogenous suspension at plating and allow the cells to sit for 30-45 min before putting into incubator</li> <li>B. Pipette carefully</li> <li>C. Pipette up and down a few times before collecting sample</li> <li>D. Carefully tap the side of the plate to remove bubbles</li> </ul>
Low reading in wells	<ul style="list-style-type: none"> <li>A. Reading time is too short</li> <li>B. Samples overheated/dried</li> <li>C. The substrate is too cold</li> </ul>	<ul style="list-style-type: none"> <li>A. Increase the integration time</li> <li>B. Keep the plate away from heat source</li> <li>C. Warm up the substrate to room temperature before use</li> </ul>
Sample signal is too strong	<ul style="list-style-type: none"> <li>A. Cell density was too high</li> <li>B. Insufficient heat inactivation of endogenous alkaline phosphatase activity</li> </ul>	<ul style="list-style-type: none"> <li>A. Reduce cell plating density</li> <li>B. Correct the duration or temperature of heat inactivation step</li> </ul>
Poor control curve/signal	<ul style="list-style-type: none"> <li>A. Control compound degraded</li> <li>B. Pipetting error</li> <li>C. Splashing of sample</li> <li>D. Volume carry-over during dilution</li> </ul>	<ul style="list-style-type: none"> <li>A. Avoid free-thaw of positive control</li> <li>B. Check pipette volume</li> <li>C. Dispense carefully</li> <li>D. Use new tip for each pipetting</li> </ul>



## References

1. Schaap, F.G., Trauner, M., and Jansen, P.L.M. Bile acid receptors as targets for drug development. *Nat. Rev. Gastroenterol. Hepatol.* **11**, 55-67 (2014).
2. Kawamata, Y., Fujii, R., Hosoya, A., et al. A G protein-coupled receptor responsive to bile acids. *J. Biol. Chem.* **278(11)**, 9435-9440 (2003).
3. Pols, T.W.H., Noriega, L.G., Nomura, M., et al. The bile acid membrane receptor TGR5 as an emerging target in metabolism and inflammation. *J. Hepatol.* **54(6)**, 1263-1272 (2011).
4. Perino, A. and Schoonjans, K. TGR5 and immunometabolism: Insights from physiology and pharmacology. *Trends Pharmacol. Sci.* **36(12)**, 847-857 (2015).
5. Duboc, H., Taché, Y., and Hofmann, A.F. The bile acid TGR5 membrane receptor: From basic research to clinical application. *Dig. Liver Dis.* **46(4)**, 302-312 (2014).
6. Evans, K.A., Budzik, B.W., Ross, S.A., et al. Discovery of 3-aryl-4-isoxazolecarboxamides as TGR5 receptor agonists. *J. Med. Chem.* **52(24)**, 7962-7965 (2009).



### 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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