Bile Acid Nuclear Receptor FXR (NR1H4) Reporter Assay Kit

Item No. 601790

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

TABLE OF CONTENTS

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
601791	FXR Reverse Transfection Strip Plate	1 plate	-20°C
601792	XL335 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. HEK293T/17 cells, available from ATCC
2. Culture medium used for maintenance of the cells (DMEM with 10% FBS and 1X penicillin-streptomycin)
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

INTRODUCTION

Background

Bile acids are a diverse class of amphipathic molecules originally formed in liver and modified by microorganisms in the intestine.[1] 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 other bile acids, glucose homeostasis, and energy expenditure.[1,2] Several nuclear receptors, including the farnesoid X receptor (FXR/NR1H4), pregnane X receptor (PXR), vitamin D_3 receptor (VDR), and constitutive androstane receptor (CAR), as well as the G protein-coupled receptor TGR5/GP-BAR1, interact with certain bile acids and carry out some of their signaling pathways and processes.[1] FXR was the first identified bile acid receptor and is the most important for bile acid signaling among these nuclear receptors. There are at least four isoforms of FXR formed by alternative splicing, and they exhibit tissue-specific distribution.[3] The ligand-activated FXR functions as a transcription factor both as a monomer and as a heterodimer with retinoic acid receptor α (RXRα) to regulate the expression of various genes, including the bile salt export pump (BSEP), which is upregulated by the activation of FXR.[3-5] The FXRα2/FXRα1(-) is the predominant isoform that regulates BSEP expression.[3,5]

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 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 is increased dramatically compared to solution-phase transfection, enhancing both the transfection efficiency and co-transfection efficiency for multiple plasmids.

Cayman's Bile Acid Nuclear Receptor FXR (NR1H4) Reporter Assay Kit consists of a 96-well plate coated with a transfection complex containing DNA constructs for expressing FXR and a BSEP promoter-regulated secreted alkaline phosphatase (SEAP) reporter (FXR Reverse Transfection Strip Plate). Cells grown on the transfection complex will express FXRα2 inside the cells within 24 hours from an engineered plasmid construct. Binding of agonists to FXR triggers its transcription factor activity. Activation of the BSEP promoter on the reporter construct by FXR results in the expression of SEAP, which is secreted into the cell culture medium. Aliquots of media are collected 6-24 hours after stimulation, and SEAP activity is measured following addition of a luminescence-based alkaline phosphatase substrate (SEAP Substrate (Luminescence)). The kit is easy to use and can be readily applied to high-throughput screening for therapeutic compounds regulating the activation of FXR. A selective synthetic agonist, XL335, is included in the kit for use as a positive control.[6] The kit provides sufficient reagent to measure SEAP activity at three time points using the three included 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 XL335 Positive Control (Item No. 601792), 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 amount of XL335 Positive Control provided is sufficient to run a full dose-response curve with replicates up to 10 µM.

Addition of Cells to the Reverse Transfection Plate

IMPORTANT

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

1. Remove the FXR Reverse Transfection Strip Plate (Item No. 601791) from the freezer and allow it to equilibrate to room temperature within the sealed bag.
2. After the plate has reached room temperature and before opening the bag, clean the bag with 70% alcohol and place the plate inside the hood.
3. Seed HEK293T/17 cells at a density of 30,000-50,000 cells/well in 200 µl 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 incubator with 5% CO_2 and incubate for 18-24 hours.

*NOTE: If the whole plate will not be completely used within one experiment, remove the number of strips needed and place the remaining strips back in the bag. 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 the 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 cells with 150 µl of pre-warmed serum-free stimulation medium per well.
3. Prepare test compounds at 4X the desired final concentration in serum-free stimulation media and pipette 50 µl into the assigned wells.
4. For untreated control cells, pipette 50 µl of stimulation media per well.
5. For positive control wells, dilute the provided 10 mM XL335 Positive Control 1:2,500 in the stimulation medium and add 50 µl per positive-control well.
6. Return the cells to incubator at 37°C with 5% CO_2 and incubate for 16-18 hours

NOTE: At 1 µM, XL335 Positive Control typically induces a >3-fold increase in SEAP activity in 16-18 hours over untreated control. Prepare aliquots of XL335 Positive Control 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, allow the SEAP Substrate (Luminescence) (Item No. 600183) to equilibrate to room temperature.

1. After stimulation with test compounds and controls, use a multi-channel pipette to gently pipette up and down a few times, collect 10 µl of media from each well, and pipette it into the corresponding well of the 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 the 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 to room temperature.
5. Add 50 µl SEAP Substrate to each well, shake/tap briefly to mix, and incubate 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 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 XL335 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) 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

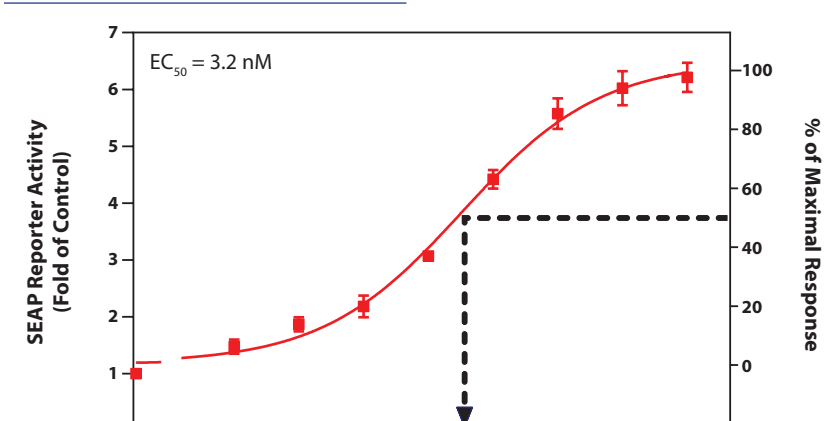


Figure 1. SEAP activity in HEK293T/17 cells transiently transfected with FXR in response to XL335 stimulation. HEK293T/17 cells were plated on an FXR Reverse Transfection Strip Plate at a density of 40,000 cells/well and incubated overnight. The next day, cells were replenished with fresh medium and treated with serial dilutions of XL335 Positive Control in serum-free medium. After 16 hours of stimulation, 10 µl of culture media was collected from each well and the SEAP activity of each sample was measured as described. The calculated EC_{50} value from the fitted curve is 3.2 nM and the Z' value is >0.8.
NOTE: The fold of stimulation, Z´ value, and calculated EC_{50} may vary with cell lines, cell passages, and culture conditions.

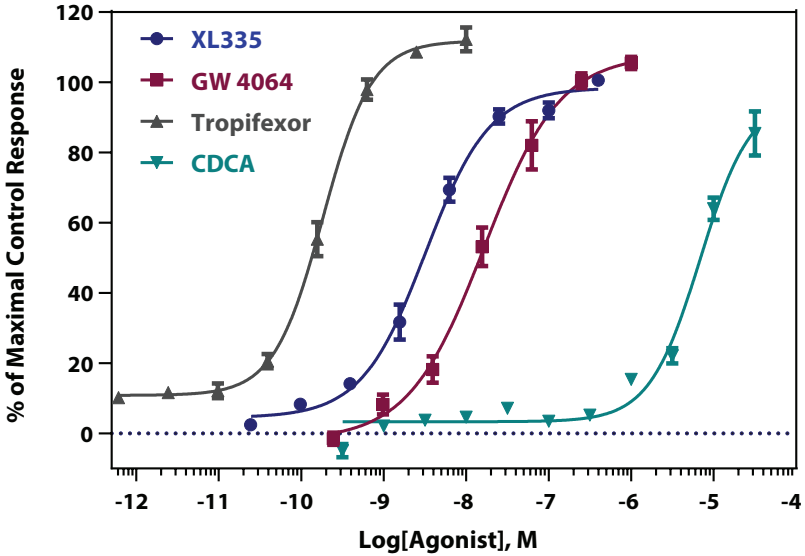


Figure 2. Validation of the FXR Reporter Assay Kit with different classes of agonists. In addition to XL335 Positive Control, three known FXR receptor agonists were examined in the FXR reporter assay, including synthetic non-steroidal compounds and a bile acid. HEK293T/17 cells transfected on strip well plates were treated with serial dilutions of XL335 Positive Control, GW 4064 (Item No. 10006611), tropifexor (Item No. 25748), and chenodeoxycholic acid (CDCA; Item No. 10011286). Media samples were collected and analyzed as described. The reporter activities were normalized to the responses by XL335 Positive Control on the same plate. All examined compounds appeared to be full agonists. The calculated EC_{50} values for XL335, GW 4064, tropifexor, and CDCA are 3.2, 16.5, 0.18, and 6,900 nM, respectively.

RESOURCES

Troubleshooting

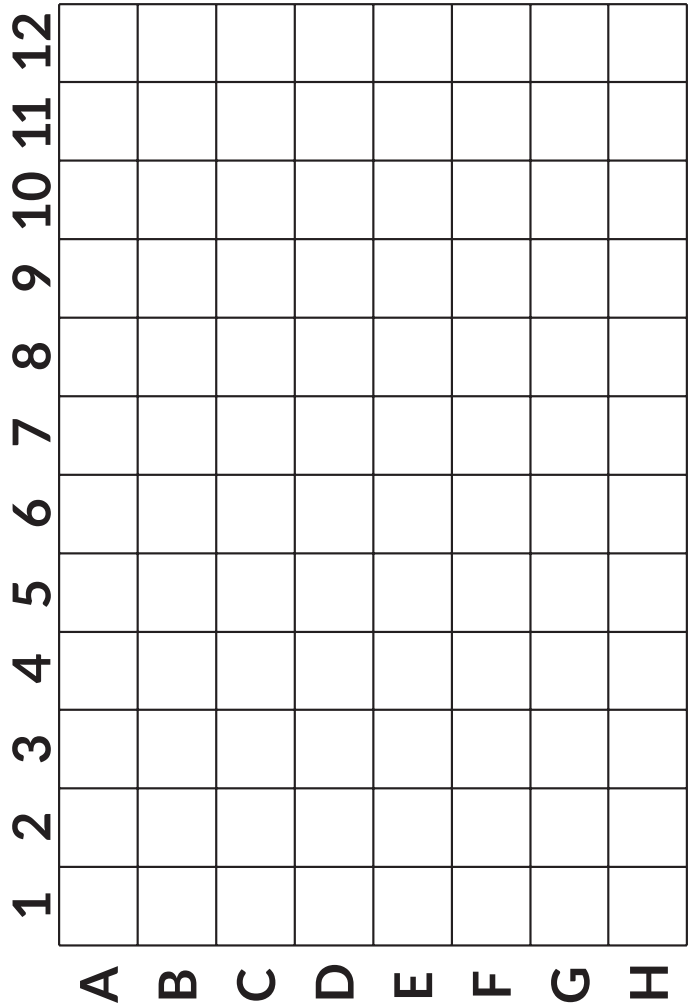
Problem	Possible Causes	Recommended Solutions
Dispersion of replicates or erratic response curve of test compounds	A. Uneven cell distribution B. Poor pipetting C. Not well mixed when sampling D. Bubble in assay wells	A. Make sure cells are in homogenous suspension at plating and allow the cells to sit for 30-45 min before placing into the 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 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 freeze-thaw of positive control B. Check pipette volume C. Dispense carefully D. Use new tip for pipetting into each well

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(1)**, 55-67 (2014).
2. Di Ciaula, A., Garruti, G., Baccetto, R.L., *et al.* Bile acid physiology. *Ann. Hepatol.* **16(Suppl. 1)**, s4-s14 (2017).
3. Zhang, Y., Kast-Woelbern, H.R., and Edwards, P.A. Natural structural variants of the nuclear receptor farnesoid X receptor affect transcriptional activation. *J. Biol. Chem.* **278(1)**, 104-110 (2003).

4 Ananthanarayanan, M., Balasubramanian, N., Makishima, M., *et. al.* Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. *J. Biol. Chem.* **276(31)**, 28857-28865 (2001).

5. Song, X., Chen, Y., Valanejad, L., *et al.* Mechanistic insights into isoform-dependent and species-specific regulation of bile salt export pump by farnesoid X receptor. *J. Lipid Res.* **54(11)**, 3030-3044 (2013).
6. Flatt, B., Martin, R., Wang, T.L., *et al.* Discovery of XL335 (WAY-362450), a highly potent, selective, and orally active agonist of the farnesoid X receptor (FXR) *J. Med. Chem.* **52(4)**, 904-907 (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.