CYP2B6 Induction Reporter Assay Kit

Item No. 600680

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

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GENERAL INFORMATION

Materials Supplied

Item Number	Item Name	100 Tests Quantity/Size	Storage Temperature
600682	CYP2B6 Reporter Reverse Transfection Strip Plate	1 plate	-20°C
600681	CITCO Positive Control (10 mM)	1 vial/10 µl	-20°C
600183	SEAP Substrate (Luminescence)	1 vial/15 ml	4°C
600272	96-Well Solid Plate (white) with lid	3 plates	Room temperature

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.

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

Remove the CITCO Positive Control from the kit and store at -20°C. Store the CYP2B6 Reporter Reverse Transfection Strip Plate at -20°C. The SEAP Substrate should be stored at 4°C. The kit will perform as specified if used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. HepG2 cells or C3A cells; both cell lines can be obtained from ATCC
2. Culture medium used for the cells (MEM containing 10% Fetal Bovine Serum (FBS))
3. A plate reader capable of measuring luminescence
4. Adjustable pipettes and a repeating pipettor
5. An incubator set at 65°C
6. Penicillin-Streptomycin (100X) (Invitrogen 15140-122)

INTRODUCTION

Background

The cytochrome P450s (CYPs) are heme-thiolate monooxygenases that metabolize a wide range of endogenous compounds and xenobiotics, such as pollutants, environmental compounds, and drugs.[1] These enzymes are essential for the metabolism of many medications and their induction is one of the factors that can affect the pharmacokinetics of a drug upon multiple dosing. It has been discovered that CYP2B6 plays a critical role in the biotransformation of several clinically important drugs.[2] Moreover, 2B6 is a CYP isozyme highly inducible by an array of structurally diverse chemicals.[3,4]

Xenobiotic induction of human CYP2B genes is regulated at the transcriptional level through interaction with nuclear receptors. Among them, human constitutive androstane receptor (hCAR)-mediated induction involves two different mechanisms: receptor activation and nuclear translocation. A number of splice variants of the hCAR gene have been identified. The hCAR3 variant is efficiently activated by all the known hCAR activators including CITCO {6-(4-clorophenyl)imidazo[2,1-b]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl) oxime} and Phenobarbital.[3,4]

Early screening of a drug candidate's potency to induce or inhibit CYP2B6 is a critical step in preventing potential drug failure due to toxicity or low efficacy at the later stages of drug development. Primary human hepatocytes are commonly used for assessment of CYP2B6 induction. However, their limited supply and significant donor-to-donor variation complicate their application in early drug discovery. Consequently, there is a need to generate human hepatocyte-like cells which are unlimited in supply and provide the regulatory pathways involved in drug metabolism. Cell-based reporter assays that typically involve cultured cell lines transfected with a reporter gene construct containing a promoter/enhancer sequence of the enzymes have provided a means of high-throughput enzyme induction assessment. Therefore, a cell-based reporter assay to evaluate hCAR3-mediated CYP2B6 induction represents a novel assay for the discovery of hepatic CYP inducers.

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 CYP2B6 Induction Reporter Assay Kit consists of a 96-well plate coated with a transfection complex containing a Secreted Alkaline Phosphatase (SEAP) reporter regulated by the human CYP2B6 gene promoter. The complex also contains two nuclear receptor expression constructs, human Constitutive Androstane Receptor 3 (hCAR3) and human Hepatocyte Nuclear Factor 4α (HNF4α).[5] Cells grown on the CYP2B6 Reverse Transfection Strip Plate will introduce the reporter gene and express CAR3 and HNF4α. Binding of inducer-activated receptor and transcription factors to the CYP2B6 promoter initiates a signal resulting in expression of SEAP, which is secreted into the cell culture medium. Aliquots of medium are removed at time intervals beginning at about 24 hours and SEAP activity is measured simply by adding a luminescence-based alkaline phosphatase substrate provided in the kit. The kit is simple to use and can be easily adapted to high-throughput screening for potential CAR-mediated CYP2B6 inducers. A known CYP2B6 inducer, CITCO, is included in the kit for use as a positive control. The kit provides sufficient reagents to measure SEAP activity at three time points using the white 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 CITCO 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. Record the contents of each well on the template sheet provided on page 13.

Addition of Cells to the Reverse Transfection Plate

IMPORTANT

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

1. Remove the CYP2B6 Reporter Reverse Transfection Strip Plate (Item No. 600682) 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 35,000-45,000 cells/well in 200 µl of culture medium. Place the plate in a 37°C incubator and incubate overnight or up to 48 hours.

Cell Stimulation

1. After 24 hours of incubation, aspirate the culture media from each well.
2. Add 100 µl of culture media to each well.
3. Prepare test compounds at 2X the desired final concentration in the above culture medium and pipette 100 µl to the assigned wells. Wells containing untreated cells receive 100 µl of culture medium only. For positive controls using the provided CITCO, dilute the CITCO Positive Control (Item No. 600681) 1:1,000 in the culture medium and add 100 µl to corresponding wells. At this concentration, CITCO induces a 5-10-fold increase in SEAP activity, depending on the cell type and stimulation time used.

NOTE: It is recommended the assay be performed when the cells are near confluency. For prolonged incubations, test compounds may need to be replenished with media change.

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 the refrigerator and allow to equilibrate to room temperature.

1. After 24-72 hours of stimulation with test compounds and CITCO, collect 10 µl of medium from each well and transfer into a 96-Well Solid Plate (white) with lid (Item No. 600272) inside a culture hood. The Reverse Transfection Strip Plate must be returned into the incubator if further sample collection will be done. *NOTE: It is recommended to collect samples for assay after 24, 48, and 72 hours of stimulation. Unstable test compounds may be replenished daily right after sample collection by media change.*
2. Cover the white plate with the lid and 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.
3. Remove the plate from the 65°C incubator and allow to equilibrate to room temperature.
4. Add 50 µl of substrate to each well, shake briefly, and incubate the plate at room temperature for 30 minutes.
5. Read the plate with a plate reader capable of detecting a luminescent signal.

NOTE: The plate should be read immediately after 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

According to the industry guidelines, a compound that produces a change that is equal to or greater than 40% of the positive control can be considered as an enzyme inducer *in vitro* and therefore *in vivo* evaluation is warranted.

For each compound, calculate the % Response as follows:

$$\left[\frac{\text{(SEAP activity (RLU) of test drug treated cells) - (SEAP activity (RLU) of untreated cells)}}{\text{(SEAP activity (RLU) of CITCO treated cells) - (SEAP activity (RLU) of untreated cells)}}\right] \times 100$$

Performance Characteristics

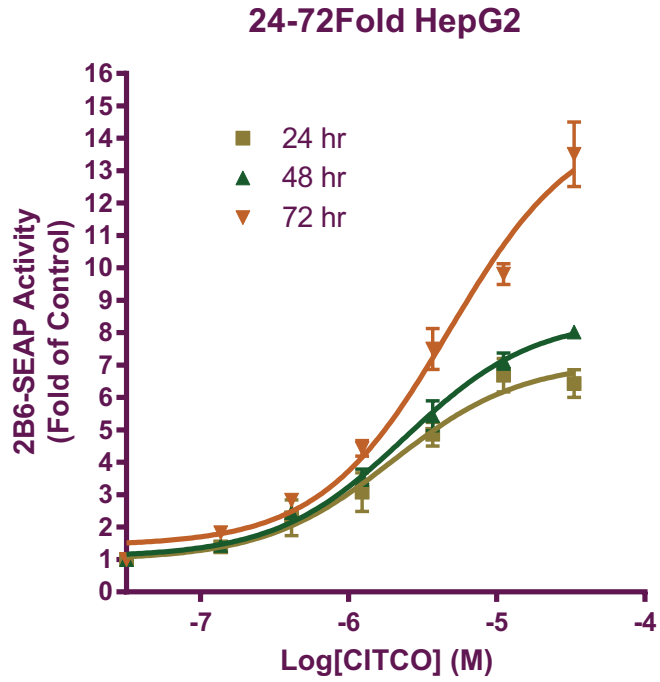
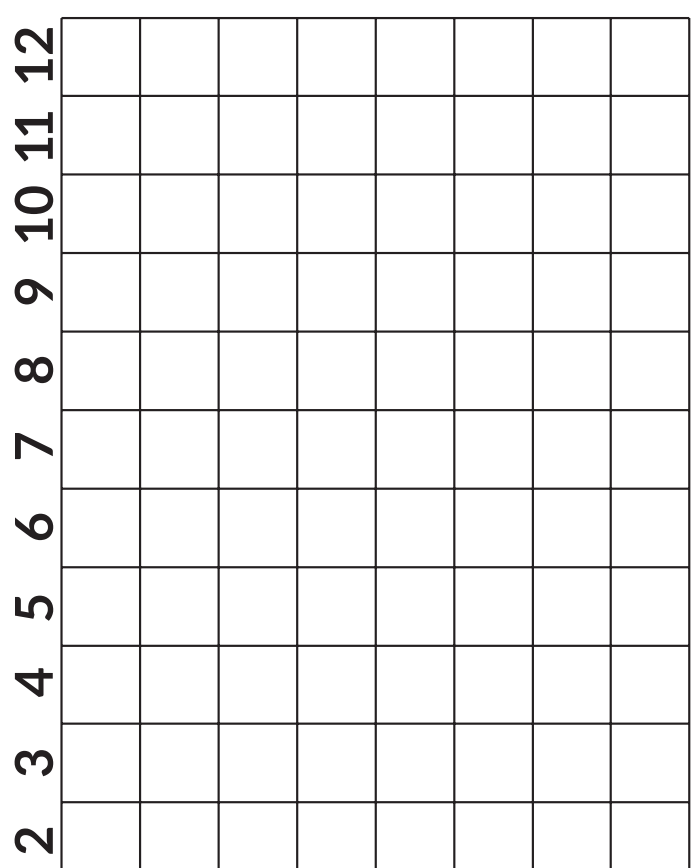


Figure 1. CAR3-mediated CYP2B6 SEAP reporter activity in HepG2 cells in response to CITCO stimulation. HepG2 cells were plated on a CYP2B6 SEAP Reporter Reverse Transfection Strip Plate at a density of 35,000 cells/well. At 24 hours after plating, cells were treated with different doses of CITCO as indicated on the x-axis. After 24, 48, and 72 hours of stimulation, 10 µl of culture media was removed from each well and assayed for SEAP activity according to the protocol described on pages 8-9. Z' is ≥0.7 at 24, 48, and 72 hours using 10 µM CITCO as positive control. The EC_{50} values were between 2.0 and 4.8 µM.

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	Unequeal 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 or treatment was too long	Plate cells more sparsely or decrease treatment time
Decrease in SEAP activity at high doses of compound	Cytotoxicity at high doses of compound	Use compound at lower doses



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3. Wang, H. and Tompkins, L.M. CYP2B6: New insights into a historically overlooked cytochrome P450 isozyme. *Curr. Drug Metab.* **9(7)**, 598-610 (2008).
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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.