CYP2C9 Induction Reporter Assay Kit

Item No. 601120

Customer Service 800.364.9897 * **Technical Support** 888.526.5351
www.caymanchem.com

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	100 Tests Quantity/Size	Storage
601121	CYP2C9 Reporter Reverse Transfection Strip Plate	1 plate	-20°C
601122	Dexamethasone 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 laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

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 Dexamethasone Positive Control from the kit and store at -20°C. Store the CYP2C9 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. C3A cells or HepG2 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. In particular, CYP2C9 is the principal member of the CYP2C subfamily of P450 enzymes in the human liver. It is responsible for metabolizing approximately 20% of all prescribed drugs, including the diabetic agent tolbutamide, the anticonvulsant phenytoin, the anticoagulant warfarin, numerous anti-inflammatory drugs such as ibuprofen and diclofenac, the antihypertensive losartan, and many others.[2] Xenobiotic induction of the human CYP2C9 gene is regulated at the transcriptional level through interaction with nuclear receptors such as PXR, CAR, HNF4α, and GRα. Strong induction of CYP2C9 is mediated by the human Glucocorticoid Receptor (GRα) directly binding to the glucocorticoid response element (GRE) in the promoter region of the gene.[3,4] Early screening of drug candidates for induction or inhibition of CYP2C9 is a critical step in preventing potential drug failure due to toxicity or low efficacy at the later stages of drug development. 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 hGR-mediated CYP2C9 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 CYP2C9 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 CYP2C9 gene promoter. The complex also contains two additional expression constructs, the transcriptionally active isoform of the human Glucocorticoid Receptor (GRα) and human Hepatic Nuclear Factor 4α (HNF4α). Cells grown on the CYP2C9 Reporter Reverse Transfection Strip Plate will introduce the reporter gene and express GRα as well as HNF4α. Binding of inducer-activated transcription factors to the CYP2C9 promoter initiates a signal resulting in expression of SEAP, which is secreted into the cell culture medium. Aliquots of medium are collected 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 GR-mediated CYP2C9 inducers. A known CYP2C9 inducer, dexamethasone, 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 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 dexamethasone 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 15.

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 unopened CYP2C9 Reporter Reverse Transfection Strip Plate (Item No. 601121) 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 40,000-60,000 cells/well in 150 µl of culture medium. Place the plate in a 37°C cell incubator and incubate for 24 hours.

Cell Stimulation

1. After 24 hours of incubation, check the cells for confluency.
2. Prepare test compounds at 4X the desired final concentration in culture medium, as prepared above, and pipette 50 µl to the assigned wells. Wells containing untreated cells receive 50 µl of culture medium only. For positive controls using the provided dexamethasone, dilute the 10 mM Dexamethasone Positive Control (Item No. 6001122) 1:10,000 in the culture medium and add 50 µl to corresponding wells. At this concentration, dexamethasone induces a 20- to 100-fold increase in SEAP activity, depending on the cell type and stimulation.
3. Return the plate to 37°C incubator.

NOTE: It is recommended the assay be performed when the cells are near confluency. For prolonged incubations, test compounds may need to be replenished daily 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 dexamethasone, 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.*
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 evaluation *in vivo* is warranted.

For each compound, calculate the % Response as follows:

% Response at X Concentration =

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

Performance Characteristics

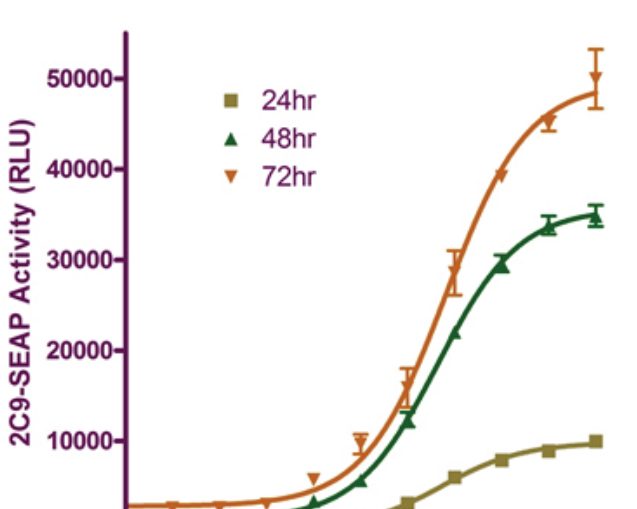


Figure 1. GRα-mediated CYP2C9 SEAP reporter activity in HepG2 cells in response to dexamethasone stimulation. HepG2 cells were plated on a CYP2C9 Reporter Reverse Transfection Strip Plate at a density of 50,000 cells/well. At 24 hours after plating, cells were treated with different doses of dexamethasone as indicated above 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 page 9. Z'=0.78 at 48 hours.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of replicates	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 test compounds	Unequal 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 (decrease treatment time)
Decrease in SEAP activity at high doses of compound	Cytotoxicity at high doses of compound	Use compound at low doses

References

1. Tanaka, E. Clinically important pharmacokinetic drug-drug interactions: Role of cytochrome P450 enzymes. *J. Clin. Pharm. Ther*. **23(6)**, 403-416 (1998).
2. Goldstein, J.A. and de Morais, S.M. Biochemistry and molecular biology of the human CYP2C subfamily. *Pharmacogenetics* **4(6)**, 285-299 (1994).
3. Gerbal-Chaloin, S., Daujat, M., Pascussi, J.M., *et al*. Transcriptional regulation of CYP2C9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. *J. Biol. Chem*. **277(1)**, 209-217 (2002).
4. Dvorak, Z. and Pavek, P. Regulation of drug-metabolizing cytochrome P450 enzymes by glucocorticoids. *Drug Metab. Rev*. **42(4)**, 621-635 (2010).

Related Products

CYP1A1/2 Induction Reporter Assay Kit - Item No. 600670
CYP2B6 Induction Reporter Assay Kit - Item No. 600680
EP_2 Receptor (rat) Reporter Assay Kit - Item No. 600340
EP_4 Receptor (rat) Activation Assay (cAMP) - Item No. 600410
EP_4 Receptor (rat) Reporter Assay Kit - Item No. 600350
Melanocortin-3 Receptor Reporter Assay Kit - Item No. 600180
Melanocortin-4 Receptor Reporter Assay Kit - Item No. 600190
Orexin Receptor 1 Reporter Assay Kit - Item No. 600240
Orexin Receptor 2 Reporter Assay Kit - Item No. 600250

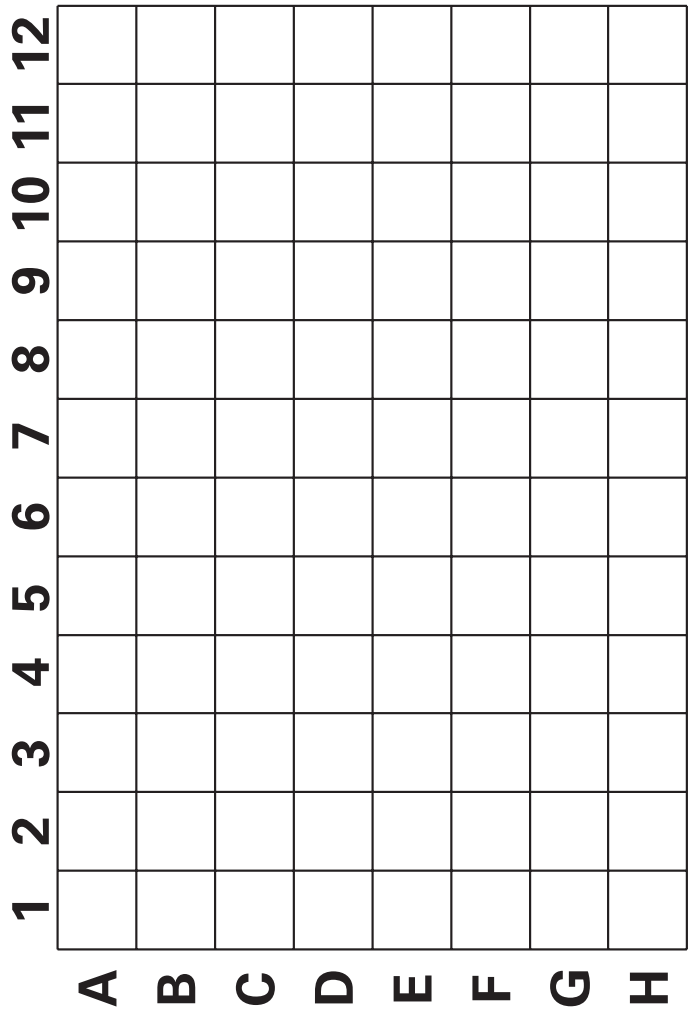
Warranty and Limitation of Remedy

Cayman Chemical Company makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.



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