CYP3A4 Induction STEP Reporter Assay Kit (Luminescence)

Item No. 600270

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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
N/A	CYP3A4 STEP Strip Plate	1 plate	-20°C
600271	Rifampicin Positive Control	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) 975-3999. 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 Rifampicin Positive Control from the kit and store at -20°C. Store the CYP3A4 STEP 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 repeat pipettor
5. An incubator set at 65°C
6. Penicillin-Streptomycin (100X) (Invitrogen 15140-122)

INTRODUCTION

Background

The cytochrome P450s (CYPs) are heme-thiolate monoxygenases 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. This can result in pharmacokinetic drug-drug interactions with co-administrated drugs, causing potential therapeutic failures. Of more than 50 known CYP enzymes, five of them, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 metabolize more than 90% of drugs. CYP3A4 is a highly inducible human CYP which metabolizes approximately 70% of marketed drugs and endogenous compounds.[2,3] Early screening of a drug candidate's potency to induce or inhibit CYP3A4 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 CYP3A4 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.[4]

About This Assay

STEP (Surface Transfection and Expression Protocol) is a novel, patented, solid phase transient transfection technology that overcomes many of the disadvantages of other transfection approaches. STEP is exclusively licensed to Originus, Inc. from the University of Michigan (US patents 6897067, 6902933, and 7056741). In this method, a proprietary STEP transfection complex containing DNA and an optimized mixture of native and recombinant 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 wells of the plate and allowed to grow over the STEP coating. Using this method, the probability of receptor-mediated endocytosis of the STEP DNA complex increases dramatically compared to solution-phase transfection, enhancing both the transfection efficiency and the co-transfection efficiency for multiple plasmids.

Cayman's CYP3A4 Induction STEP Reporter Assay Kit (Luminescence) consists of a 96-well plate coated with a STEP complex containing a Secreted Alkaline Phosphatase (SEAP) reporter regulated by the human CYP3A4 gene promoter (see Figure 1, on page 7). The complex also contains two nuclear expression constructs, Pregnane X Receptor (PXR) and Hepatocyte Nuclear Factor-4α (HNF-4α). Cells grown on the CYP3A4 STEP Strip Plate will introduce the reporter gene and express the PXR and HNF-4 factors. Binding of inducer-activated transcription factors to the CYP3A4 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 CYP3A4 inducers. A known CYYP3A4 inducer, rifampicin, 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.

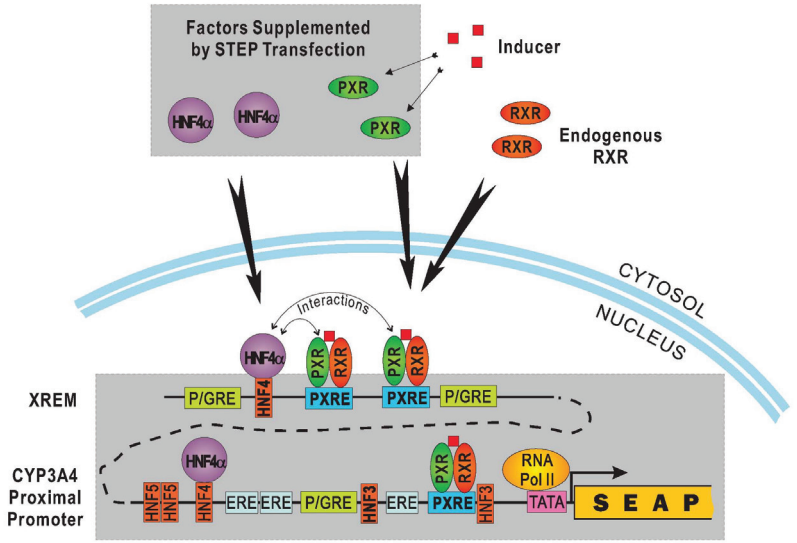


Figure 1. Diagram of CYP3A4 SEAP reporter introduced by STEP. The STEP plates are coated with a transfection complex containing a SEAP reporter gene controlled by human CYP3A4 promoter sequences including the upstream xenobiotic-response enhancer module (XREM). Selected transcription factor interacting sites are shown in the diagram above. Among them, the HNF-4α and PXRE sites are the most critical ones for the induction of the CYP3A4 gene. Optimal amounts of expression constructs for PXR and HNF-4α are also included in the STEP complex since PXR levels are insufficient in most immortalized hepatic cell lines such as HepG2 and its derivatives (*e.g.*, C3A cells) whereas the HNF-4α level may vary under culture conditions. The superior co-transfection efficiency of STEP technology is crucial for the robustness and consistency of this CYP3A4 reporter gene assay. The binding of inducers, which can be the test compounds or their metabolites, to the PXR leads to the association of the activated receptor with the PXRE on the reporter construct inside the nucleus. The expression of the SEAP reporter gene is then triggered by the interactions among various transcription factors including HNF-4α and PXR/RXR, as well as the RNA polymerase holoenzyme.

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 Rifampicin 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 STEP 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 CYP3A4 STEP Strip Plate 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 20,000-40,000 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 24-48 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 Rifampicin, dilute the Rifampicin Positive Control (Item No. 600271) 1:1,000 in the culture medium and add 100 µl to corresponding wells. At this concentration, Rifampicin induces a 15-25 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.

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 refrigerator and allow to equilibrate to room temperature.

1. After 24-72 hours of stimulation with test compounds and Rifampicin, 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 STEP 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 *in vivo* evaluation is warranted.

For each compound, calculate the % Response as follows:

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

Performance Characteristics

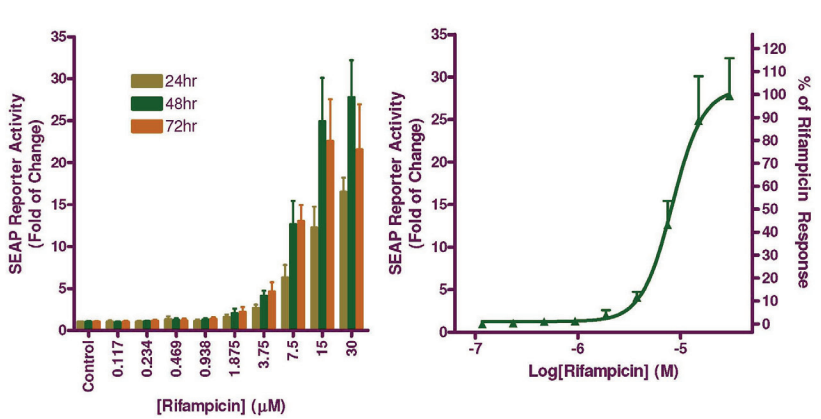


Figure 2. CYP3A4 SEAP reporter activity in C3A cells in response to rifampicin stimulation. C3A cells were plated on a CYP3A4 SEAP Reporter STEP Strip plate at a density of 20,000 cells/well. At 24 hours after plating, cells were treated with different doses of rifampicin as indicated above on the x-axis. 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 10. Maximum induction of the reporter gene by rifampicin was achieved after incubating for 48 hours at 15 µM or above. The luminescence signal further increased in samples from 72 hours stimulation; however, the fold change over the media control remained at approximately the same level. The right panel shows the dose-response curve of CYP3A4 reporter induction by rifampicin at 48 hours.

RESOURCES

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 compound treatments	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. Bibi, Z. Role of cytochrome P450 in drug interactions. *Nutr. Metab.* **5(27)**, (2008).
2. Yang, L.-Q., Li, S.-J., Cao, Y.-F., *et al.* Different alterations of cytochrome P450 3A4 isoform and its gene expression in livers of patients with chronic liver diseases. *World J. Gastroenterol.* **9(2)**, 359-363 (2003).
3. Cheng, Q., Sohl, C.D., and Guengerich, F.P. High-throughput fluorescence assay of cytochrome P450 3A4. *Nat. Protoc.* **4(9)**, 1258-1261 (2009).
4. Ripp, S.L., Mills, J.B., Fahmi, O.A., *et al.* Use of immortalized human hepatocytes to predict the magnitude of clinical drug-drug interactions caused by CYP3A4 induction. *Drug Metab. Dispos.* **34(10)**, 1742-1748 (2006).

Related Products

Adipogenesis Assay Kit - Item No. 10006908
Adipolysis Assay Kit - Item No. 10009381
EP_2 Receptor (rat) STEP Reporter Assay Kit (Luminescence) - Item No. 600340
EP_4 Receptor (rat) STEP Reporter Assay Kit (Luminescence) - Item No. 600350
LDL Uptake Cell-Based Assay Kit - Item No. 10011125
Melanocortin-3 Receptor STEP Reporter Assay Kit - Item No. 600180
Melanocortin-4 Receptor STEP Reporter Assay Kit - Item No. 600190
Orexin Receptor 1 STEP Reporter Assay Kit - Item No. 600240
Orexin Receptor 2 STEP Reporter Assay Kit - Item No. 600250

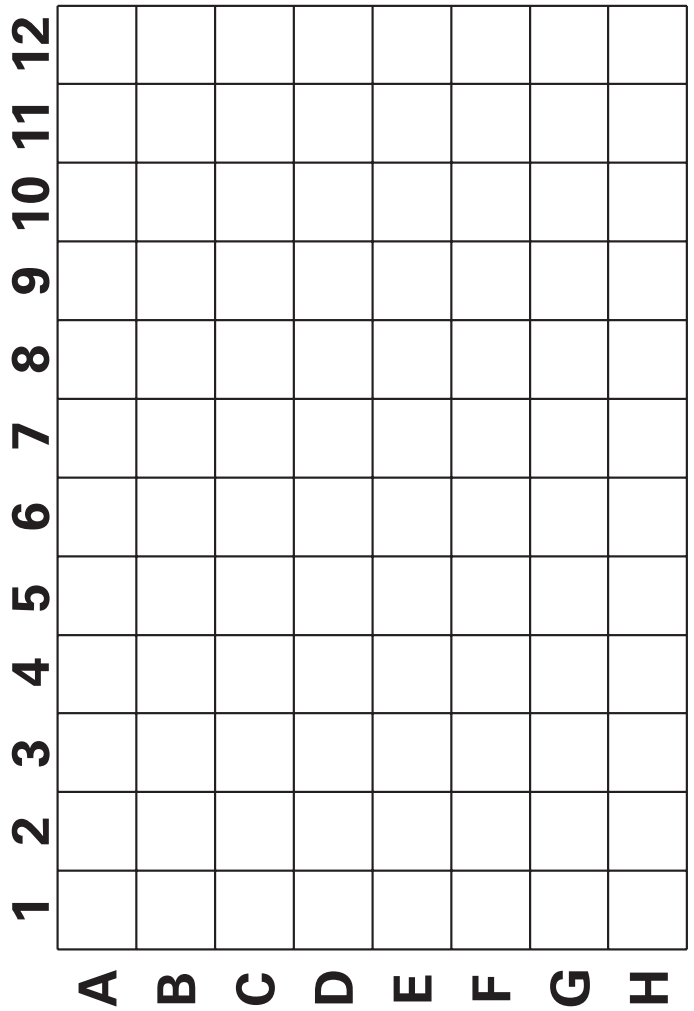
Warranty and Limitation of Remedy

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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