

Assay Methods

Protocol: Fresh Human Hepatocyte Cytochrome P450 Induction Assays

The cellular content of cytochromes P450 can be induced several fold by pre-treatment with drugs and other xenobiotics. Drug-mediated induction of P450 can result in increased metabolism of other drugs or itself, leading to potentially harmful drug interactions and/or drug tolerance. Freshly isolated human hepatocytes provide an excellent in vitro means for predicting P450 induction potential by drug candidates.

The following protocol describes a method for treating plated human hepatocytes with "classical" P450 inducers (i.e. Rifampicin-CYP3A4, 2C9/19; Phenobarbital-CYP2B6, 3A4; and β -Naphthoflavone-CYP1A2) and analysis of results. The same general protocol can be followed when testing the induction potential of new chemical entities (NCEs).

Table 1: Chemicals and Suppliers

Chemical Reagent	Supplier (Catalog No.)
Rifampicin (RIF)	Sigma Chemical Co. (R-3501)
β -naphthoflavone (β -NF)	Sigma Chemical Co. (N-3633)
Phenobarbital (PB)	Sigma Chemical Co. (P-5178)
BD™ Hepatocyte Culture Media Kit	BD Biosciences (355056)
Dimethyl Sulfoxide (DMSO)	JT Baker (T15599)
KHB Buffer	Sigma Chemical Co. (K-3753)
Phenacetin (CYP1A2 substrate)	Sigma Chemical Co. (A-2375)
Testosterone (CYP3A4 substrate)	Sigma Chemical Co. (T-1500)
Diclofenac (CYP2C9 substrate)	Sigma Chemical Co. (D-6899)
[¹⁴ C]-Mephenytoin (CYP2C19/2B6 substrate)	GE Life Sciences. (CFA-763)
S-Mephenytoin (CYP2C19/2B6 substrate)	BD Biosciences (451032)

Table 2: P450 Inducers and Substrates/Solvents and Concentrations

Chemical	Stock Concentration	Solvent	Final Working Concentration
Rifampicin (RIF)	25 mM	DMSO	20 μ M
β -Naphthoflavone (β -NF)	25 mM	DMSO	20 μ M
Phenobarbital (PB)	0.2 M	PBS	2 mM
Testosterone	0.1 M	DMSO	200 μ M
Diclofenac	0.1 M	DMSO	100 μ M
Phenacetin	0.1 M	DMSO	100 μ M
*[¹⁴ C]-Mephenytoin	10 mM	ACN	100 μ M

* Specific Activity of 10 mM stock: 5 to 7 mCi/mmol.

Other Material and Equipment:

- Biosafety hood
- Incubator with 37°C and 95% air / 5% CO₂ at atmospheric level capacity
- Basic cell culture equipment

Fresh Plated Hepatocytes:

Plated Human Hepatocytes/
BD BioCoat™ collagen I microplates

- 6-well BD BioCoat collagen I plates (cat. no. 454406)
- 12-well BD BioCoat collagen I plates (cat. no. 454412)
- 24-well BD BioCoat collagen I plates (cat. no. 454424)
- 48-well BD BioCoat collagen I plates (cat. no. 454425)
- 96-well BD BioCoat collagen I plates (cat. no. 454496)

Procedure:

1. Unpack the plated hepatocytes. Follow enclosed instructions for handling requirements and maintenance until ready for further testing.
2. Incubate the plated hepatocytes for 1-2 days in a humidified 37°C incubator with 95% air / 5% CO₂ atmosphere to allow for recovery from shipping.
3. Prepare the chemical inducers (RIF, β-NF, and PB), solvent vehicle controls (DMSO for RIF and β-NF, and PBS for PB) and any test compounds (Table 3). The media/inducer preparation should be prepared fresh on the day of use.

Note: Whenever DMSO is used to dissolve test compound the final DMSO concentration should not exceed 0.1%.

Table 3: Treatment Schedule and Inducer Concentrations

Chemical Inducer	Working Concentration	P450	Total Treatment Time
Rifampicin	20 μM	3A4, 2C9, 2C19	72 hours
PB	2 mM	2B6	72 hours
β-NF	20 μM	1A2	72 hours

4. Aspirate the media from the well and replace the media containing the desired treatment condition. Use the volumes shown below (Table 4).

Table 4: Media Volumes and Cell Number/Well

Multiwell Plate	Volume of Media	Approximate Cell number/ Well (100% confluent)
6-well	2 ml	14.4 x 10 ⁵
12-well	800 μl	6 x 10 ⁵
24-well	400 μl	3 x 10 ⁵
48-well	145 μl	1.1 x 10 ⁵
96-well	50 μl	0.42 x 10 ⁵

5. Return the cells to the incubator for 24 hours.
6. Repeat steps 4 and 5 twice so that the hepatocytes have a total of 3 day exposures to the treatment conditions over a 72 hour time period (Table 4).

Sample Analysis for Enzyme Induction:

There are currently a number of methods for analyzing samples for P450 induction, e.g., protein levels by immunoblots, mRNA levels by Northern blots or RT-PCR, and P450-specific probe substrate activity.

The following are protocols for measuring specific P450 activities using probe substrates. Quantification of the P450-dependent metabolites relies on HPLC or LC-MS analysis.

1. Dissolve the P450 substrate in the appropriate volume of culture media to give a final concentration as shown below (Table 5).

Table 5: P450 Substrates, Activities, and Working Stock Concentrations

Probe Substrates	P450 Specificity	Reaction	Working Stock
Testosterone	3A4	6β-Hydroxylase	200 μM
Phenacetin	1A2	O-Deethylase	100 μM
Diclofenac	2C9	4'-Hydroxylase	100 μM
S-Mephenytoin	2C19	4'-Hydroxylase	100 μM
S-Mephenytoin	2B6	N-Demethylase	100 μM

2. Remove the cell culture media and replace it with the same volume of substrate/media mix.
3. The cells should be incubated in a 37°C incubator (with CO₂) for approximately 30 minutes to 1 hour. Additional time points may be included to establish linear conditions.
4. At the end of the incubation period, the assay is stopped by adding an appropriate volume of stop solution to the well. **Table 6** shows suitable stop solutions and volumes that are compatible with HPLC analysis.
5. Analyze samples by HPLC. Activity values can be reported as pmol product/min/mg of cell lysate or pmol product/min/million cells. Induction potential of the test compound is determined by dividing the activity of the treated group vs. the vehicle control group.

Table 6: Reaction-Stop Solutions

P450 Substrate	Stop Solution	Volume Stop Solution/ml Incubation Volume
Testosterone	100% Acetonitrile	500 µl
Phenacetin	70% Perchloric Acid	125 µl
Diclofenac	6% Acetic Acid / 94% Acetonitrile	300 µl
S-Mephenytoin	100% Acetonitrile	250 µl



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