Rat (H4IIE) and Fish (PLHC-1) Cell Line Bioassays For the Detection of Mixed Function Oxygenase Inducers: Methods Description

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Management Perspective

In vitro cell-line bioassays for the detection of mixed function oxygenase (MFO) inducers solve several practical problems associated with their whole fish counterparts. They are less costly, logistically simpler, and less labour intensive than whole fish tests. Many experiments that would be difficult using whole fish bioassays are made possible by in vitro bioassays. In vitro assays allow a higher sample throughput, as multiple tests can be run in a few micro-well plates, and most in vitro techniques can be semi-automated by means of liquid handling devices and automated plate readers. Miniaturized in vitro bioassays radically reduce the amount of sample needed for MFO determinations, which permits tests of chemicals and samples that are expensive or available only in small amounts. In vitro bioassays are also ideal for testing small fractions from chromatographic separations of environmental samples or industrial effluents. The present report describes two in vitro bioassays based on rat (H411E) and fish (PLHC-1) cell lines for the detection of MFO inducers in small volume environmental samples. The report is intended as a standard operating procedure that the researcher can use to assess the MFO inducing potency of pure chemicals, complex mixtures, or fractions from chromatographic separations.

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Introduction

In vitro assays that use either cell lines or primary cultures of liver hepatocytes have been developed to assay the presence and activity of MFO-inducing compounds. Cells that are isolated from a parent tissue and maintained in a viable state in culture without being sub-cultured are termed "primary cultures". "Cell lines" are axenic cultures that have been sub-cultured repeatedly [1] and may be finite or continuous[2]. Several readily available fish cell lines have functional P4501A oxygenase systems[3].

In vitro MFO induction bioassays solve several practical problems associated with their whole fish counterparts. They are less costly, logistically simpler, and less labour intensive than whole fish tests. In vitro assays allow a higher sample throughput, as multiple tests can be run in a few micro-well plates, and most in vitro techniques can be semi-automated by means of liquid handling devices and automated plate readers.

Many experiments that would be difficult using whole fish bioassays are made possible by in vitro bioassays. In whole fish exposures, fish are exposed to test chemicals in 1 - 2 litres of water per g of fish per day. A total of 60 - 120 L of effluent is required to expose five 3 g fish to an industrial effluent or waterborne chemical for 4 days. Miniaturized in vitro bioassays radically reduce the amount of sample needed, which permits tests with chemicals and samples that are expensive, available only in small amounts, and with small fractions from chromatographic separations of complex mixtures.

Validated in vitro MFO tests minimize the use of live fish in screening and monitoring, thus alleviating concerns about animal welfare. In vitro assays based on fish organs, isolated cells, or cell lines can provide comparable information to whole fish bioassays, if there is a mechanistic link between the in-vitro responses and known effects in live fish. In vitro experiments, however, bear little resemblance to the complexities of natural or environmental exposures. Thus, they may not account for many of the absorption, metabolic, and pharmacokinetic processes that occur in whole fish[3]. As a result, data from in vitro assays often need to be confirmed by in vivo exposures.

Early cell line bioassays for MFO inducers

The first cell line bioassays for inducers of P4501A1 were developed as analytical tools for detecting small amounts of 2,3,7,8-T_4CDD. Based on the H411E rat liver hepatoma cell line, those assays could detect as little as 10^{-14} mole/mL of 2,3,7,8-T_4CDD[4]. Casterline et al. [5] used the H411E assay to screen extracts of fresh water fish that were prepared by fractionation on silica gel. Although the fractionation scheme was not optimized for the recovery of planar halogenated hydrocarbons (HAHs), there was good agreement between the assay and the GLC-electron capture detector data. The H411E induction assay was modified so that both ethoxyresorufin-O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH) activity could be measured in suspensions of whole cells, paving the way for the eventual use of microtitre plates and assay miniaturization[6]. The modified assay was used to screen extracts of 25 fish from the Great Lakes[7]. Bioassay estimates of 2,3,7,8-T_4CDD equivalents in fish from most of those lakes differed from the GC-MS values by less than a 2 fold margin. In contrast, bioassay estimates for fish from lakes Ontario and Erie were much higher (>4 fold) than the GC-MS derived values. The authors speculated that the higher bioassay values were caused by synergistic effects or unidentified inducers.

The H411E bioassay was systematically characterized and evaluated by Tillitt et al. using EROD activity as an end point[8]. The assay's performance was validated for a set of egg

extracts spiked with 2,3,7,8-T_4CDD: regression of observed vs expected potency was 0.949. That version of the H411E assay was later used to screen extracts of eggs from colonies of Great Lakes fish eating birds, [9] and to show that TCDD-equivalents (TEQs) in chinook salmon (Oncorhynchus tshawytscha) were transferred from mother to eggs[10]. Smith et al. also used the H411E assay to estimate bioassay derived TEQs for coho and chinook salmon from the Great Lakes[11].
The Swedish Dioxin Survey's evaluation of the H411E assay also showed good agreement between bioassay and analytically derived TEQs for herring and Osprey samples[12]. However, H411E data for white fish did not compare as well with GC-MS data, possibly because of interference by unidentified planar compounds[12].
The H411E bioassay was used to compare TEQs in white suckers, Catostomus commersoni, caught near 8 pulp mills to TEQs in fish from 5 reference sites[13]. Dilutions of fish liver extracts were tested in the H411E bioassay and were also analyzed using conventional techniques. The bioassay-derived TEQs were highest at mills that combined the Kraft pulping process with chlorine bleaching. The bioassay TEQs could mostly be explained by the concentrations of PCDD-PCDF congeners, although extracts from fish downstream of 2 mills that used little chlorine caused greater induction than was predicted by PCDD/F levels, suggesting the presence of other inducers of EROD.

Fish cell lines
The use of mammalian cell cultures to predict the potency of chemicals in fish raises concerns about possible inter-class differences in the induction process. Fish cell lines may provide data that can be more realistically extrapolated to whole fish. Tritiated and photo-affinity ligands have been used to identify the Ah receptor in the rainbow trout cell lines RTH-149 [14] and RTG-2 [15], in a variety of teleost species, and in the Poeciliopsis lucida cell line (PLHC-1)[16]. Co-planar PCB-77, 3,3',4,4'-TCBP, induced EROD activity in a PLHC-1 bioassay, with a response relationship from 0.01 ③M to 0.1 ③M TCBP after which the induced activity decreased[17]. Experimental data indicated that the decrease in activity was not a result of cytotoxicity. The amount of induced protein, measured immunologically, continued to increase up to 10 ③M, suggesting that inhibition or inactivation of the enzyme might lead to underestimates of TCDD equivalence factors (TEFs) or TEQs. Direct quantification of the enzyme protein, or its mRNA, may solve this problem.

Hahn et al. [18] confirmed the usefulness of the PLHC-1 assay by assessing the ability of 2,3,7,8-T_4CDD, 3,3',4,4',5-PCB, and 3,3',4,4'-TCB to induce EROD and CYP1A protein. As in their previous experiments a decline in EROD activity was observed to occur at lower exposure concentrations compared to P4501A protein. The estimated TEFs for both congeners were about an order of magnitude less when calculated on the basis of the CYP1A protein rather than EROD activity.
Finnish researchers have used the PLHC-1 cell line to detect MFO inducers in pulp and pulp mill effluents[19]. Pulp bleached without chlorine caused the highest induction of EROD, suggesting that chlorinated compounds may not be the sole inducers.

A non-hepatoma cell line, RTL-W1, that expresses inducible EROD activity, has been developed from rainbow trout liver, [20] and was used to derive sets of TEFs for a series of PCDD/PCDF/PCB congeners[21,22]. Most of the RTL-W1-derived TEFs for PCDD/Fs were

substantially higher (2-8 x) than H411E TEFs measured in the same lab., although the rank order potencies were the same. The RTL-W1 derived TEFs correlated well with those previously reported for live fish, such as orally dosed rainbow trout[23]. In both cases, fish derived TEFs were higher than TEFs established internationally to assess dioxin risks to humans. For PCBs, H411E derived TEFs tended to be higher than those derived by means of the RTL-W1 bioassay, particularly for the non-ortho congeners, suggesting that TEFs for assessing risks to fish would be more appropriately derived from fish-cell based assays. Thus, the RTL-W1 cell line assay may be more useful than H411E assays for predicting which congeners or samples are likely to induce MFO activity in fish. Although the PLHC-1 assay may be less sensitive than the H411E and RTL-W1 assays, its maximum response signal is at least as strong as observed in the RTL-W1 assay.

In the present report we describe 2 cell line assays for the detection of MFO activity in environmental samples. One based on the H411E mammalian cell line and the other based on the PLHC-1 fish cell line.

Method Descriptions

General Cell Line Maintenance Techniques
The following techniques are applicable to both the H411E and PLHC-1 cell line bioassays. It is recommended that a count be kept of the number of passages that the cells have undergone and to be aware that the characteristics of a cell line can vary with time and passage number. The total number of cells in the 75 cm^2 culture flask should be periodically recorded at passage time. The response of the cells to standard inducers should also be recorded. If any drift is observed in the growth rate or responsiveness of the cells, a fresh working culture can be revived from the stock cultures that are stored in liquid nitrogen. The performance of the PLHC-1 cell line is known to change with passage number[18]. The optimum passage number for the EROD induction assay has yet to be determined.
Because the PLHC-1 cells grow best at high densities, a split ratio of 1:8 or less is recommended. The H411E cells are best split so that the 75 cm^2 culture flask becomes confluent in 7 days. That period can be shortened if experiments are started more frequently than weekly.

Aseptic techniques:
It is critical that strict aseptic techniques be used when working with cell lines. Refer to Freshney [1] for a detailed description of suitable aseptic protocols. Ideally traffic and external sources of contamination should be kept to a minimum in the cell culture laboratory. All work should be conducted in a laminar flow hood. Swab the work area thoroughly with 70% alcohol. Likewise swab all items that are introduced into the work area. In addition to swabbing with alcohol it is strongly recommended that the lids of all reagent bottles be wrapped in Parafilm during storage. The necks of reagent bottles are to be thoroughly swabbed with alcohol before being opened.

Examination of cultures:

Cell cultures must be routinely examined through an inverted microscope for typical/atypical growth patterns and for degree of confluence. If the cells are not growing well or if, shortly after inoculation, they are unevenly distributed across the cluster of culture wells the assay should be aborted and the cell maintenance problems solved before proceeding. Within individual wells, the PLHC-1 cells may "clump" or form ridges as part of normal growth - especially if super-confluence is promoted in the splitting/growth of cells.

Materials:

- *laminar flow hood*
- *48-well culture clusters (Costar, 3025)*
- *Trypsin-EDTA, 1x*
- *Dulbecco's Phosphate Buffered Saline pH 7.8*
- *growth medium (Dulbecco's Modified eagles Medium (DMEM) for H411E; Eagle's Minimum Essential Medium (MEM) for PLHC-1)*
- *sterile pipettes: 10- and 25 or 50- mL*
- *pipette-aid*
- *sterile 100 - 500 mL glass containers*
- *aspirator*
- *haemocytometer*
- *microscope (10 x 10 magnification)*
- *15 and 50 mL conical centrifuge tubes (sterile)*
- *clinical centrifuge*
- *Eppendorf repeater pipette with sterile 250 ③L tip*
- *vortex mixer*
- *sterile glass pipettes (10 mL)*
- *adjustable pipettes (100 - 1000 ③L) with sterile tips*

Revival of frozen stock cultures:

To revive cells that have been stored in liquid nitrogen:

1) *warm the growth medium to 37°C for H411E, or to 30°C for PLHC-1*
2) *take a cryovial of cells from the liquid nitrogen dewar and place in a beaker of water at 37®C for H411E, or at 30°C for PLHC-1*
3) *once the cells have thawed, transfer the vial to a beaker of ethanol*
4) *for H411E cells: use a sterile 2.5 mL or smaller syringe (22 ga) to transfer the cells to a 75 cm^2 culture flask (Costar, 3025) and slowly (drop wise) add 9 mL of growth medium. For PLHC-1 cells use the same technique to transfer the cells to a 25 cm^2 culture flask and slowly add 4 mL of growth medium*
5) *place culture flask in a well humidified (>90% relative humidity) incubator and grow at 37®C in 5% CO_2 atmosphere for H411E cells, or at 30 °C for PLHC-1 cells*
6) *after 24 hours, aspirate the growth medium*
7) *replace with 10 mL of fresh medium for H411E cells or 5 mL for PLHC-1 cells- this step is intended to remove the residual DMSO which was used in the cryo-preservation of the stock culture*
8) *re-incubate as in (5) and grow to confluence with periodic changes of growth medium.*

Cryo-preservation:

To prepare cells for storage in liquid nitrogen:

1) *grow cells to confluence in 75 cm^2 culture flasks (10 mL medium per flask)*
2) *aspirate the medium from the culture flask(s)*
3) *gently wash cells with 5 mL of phosphate buffered saline (PBS) - removes residual medium which would inhibit the trypsin reaction - avoid disturbing the cells (i.e. don't direct the stream of buffer directly onto the monolayer of cells)*
4) *aspirate the PBS*
5) *add 2 mL of trypsin/EDTA to each culture flask (Trypsin EDTA Solution (1x) (Sigma, T-5775))*
6) *allow the trypsin solution to cover the flask's growth surface*
7) *place flask on flat surface for 5-15 minutes*
8) *periodically rock the flask from side to side with a gentle motion; the cell monolayer will become cloudy as the cells detach; gently tap the flask(s) to dislodge the cells, cells will stream to the bottom of the flask once they are ready to harvest*
9) *stop the trypsin reaction by the addition of 5 mL of growth medium*
10) *rinse down the growth surface and suspend the H411E cells by repeated pipetting- this serves to break up cell clumps, pipette the resulting cell suspension into a 10 mL centrifuge tube; repeated pipetting is not recommended for the PLHC-1 cells as it is reported to reduce viability (Hightower, personal communication 1995). Do not force the cells off the growth surface - that can cause cell clumping*
11) *centrifuge for 5 minutes (500 g)*
12) *aspirate most of the supernatant (leave < 1 mL)*
13) *mix pellet and supernatant by flicking the tube*
14) *top up the cell suspension to 5 mL with growth medium, mix well by tube inversion*
15) *load haemocytometer with 20 ③L of cell suspension*
16) *adjust the cell concentration to $5x10^6$ cells mL^{-1} medium - the method for estimating cell numbers is described in the following section*
17) *use growth medium amended with 10% v/v DMSO to dilute the cell suspension; sterilize the DMSO amended medium by membrane filtration*
18) *mix the cell suspension*
19) *add 1 mL portions of cell suspension to 2 mL cryovials,*
20) *place the loaded vials in a foam lined box (to allow cells to cool at 1 °C per minute) and place in -80 ®C freezer*
21) *store in liquid nitrogen after the cells have frozen.*

Estimation of cell numbers:

1) *load haemocytometer with 20 ③L cell suspension*
2) *count and record the number of cells in a 1 mm^2 grid on both sides of the haemocytometer using low magnification (10x objective). Repeat the procedure until counts are consistent*
3) *calculate the average number of cells in the grid*
4) *multiply by 10^4, to obtain the cell concentration (cells per mL) multiply by volume of cell suspension to get the total cell number.*

H411E Bioassay

Cell plating (Day 1)
Materials

- *H4IIE cells grown to confluence in a 75 cm^2 cell culture flask (Costar, 3025)*
- *48 well culture clusters (Costar, CS003548)*
- *Trypsin-EDTA,1x (store in 2 mL aliquots at -20 °C)*
- *Dulbecco's Phosphate Buffered Saline pH 7.8*
- *Dulbecco's Modified Eagles Media (DMEM; Sigma, D-5921)*
- *sterile pipettes: 10- and 50- mL*
- *pipette-aid*
- *sterile 200 mL beaker*
- *aspirator*
- *haemocytometer*
- *microscope (10 x 10 magnification)*
- *15 and 50 mL centrifuge tubes (sterile)*
- *clinical centrifuge*
- *Eppendorf repeater pipette with sterile 250 ③L tip*
- *vortex mixer*
- *sterile glass pipettes (10 mL)*
- *adjustable pipettes (100 - 1000 ③L) with sterile tips*

Table 1. DMEM Components.

Component	*Volume (mL)*
1) Dulbecco's Modified Eagle's Medium (Sigma, D-5921)	*427*
2) L-glutamine (Sigma, G-6392)	*0.292 g*
3) MEM Non-Essential Amino Acids, 100x (Sigma, M-7145)	*5*
4) MEM Essential Amino Acids, 50x (Sigma, M-7020)	*10*
5) Gentamicin, 50 mg mL^{-1} (Sigma, G-1397)	*0.5*
7) Modified Eagle's Medium vitamins (Sigma, M-6895)	*7.5*
6) Fetal Bovine Serum (Sigma, F-4135)	*50*

Note: FBS is stored at -20°C. It can be bought in 100 or 500 mL quantities. Larger volumes which are less expensive can be distributed into sterile containers (50 mL centrifuge tubes recommended) as required.

DMEM is prepared aseptically and stored at 4 oC.

Phosphate buffered saline (PBS) (Sigma, D-5527): add the following ingredients to 2 L of H2O: 0.4 g KCl, 16.0 g NaCl, 0.4 g KH2PO4, and 2.3 g Na2HPO4. Adjust to pH 7.5. Filter sterilize and store at 4 oC

Procedure:

1) thaw trypsin

2) *warm trypsin, medium, and PBS to 37o C in a water bath*
3) *aspirate medium from the culture flask(s)*
4) *gently rinse cells with 5 mL of PBS;*
5) *aspirate the PBS*
6) *add 2 mL of trypsin reagent to each culture flask*
7) *rock the flask periodically to facilitate trypsin reaction*
8) *wait about 5-10 minutes for the cells to lift; the time can vary for different batches of trypsin*
9) *stop the trypsin reaction by the addition of 5 mL of DMEM*
10) *pipette the resulting cell suspension into a 10- or 15 mL centrifuge tube, as previously described*
11) *centrifuge for 5 minutes (500 g)*
12) *aspirate most of the supernatant (leave about 1 mL)*
13) *mix pellet and supernatant*
14) *top up solution to 5 mL with DMEM. For routine cell passaging and culture maintenance 1 mL of this cell suspension is added to 9 mL of medium in a fresh tissue culture flask. That is a 1:5 split ratio. The inoculated flask is incubated at 37 oC. The passage number should be recorded on the flask. It is recommended that cells be passaged every 3-4 days. The growth period can be modified by adjusting the split ratio. The number of inoculated flasks should be adjusted according to the size of the experiment that is to be set up.*
15) *use a haemocytometer, as previously described, to determine the number of cells per mL, and calculate the total number of cells in the cell suspension*
16) *determine the amount of medium required for the experiment by multiplying the number of individual wells per microtitre plate (typically 48) to be used by 500 ③L.well-1 and then by the number of plates to be used (round up for spillage)*
    - e.g.: 48 wells.plate-1 x 500 ③L.well-1 x 7 Plates = 168 mL
    - round up to 180 mL = working volume (see Table 2)
17) *calculate the number of cells required by multiplying the working volume by 105 cells.mL-1, as indicated in Table 2; in other words, each well receives 5 x 104 cells*

Table 2. Number of Cells Required for Various Numbers of Plates

Number of Plates	*Required Volume (mL)*	*Working Volume (mL)*	*Number of Cells Needed (x106)*
1	*24*	*40*	*4*
2	*48*	*70*	*7*
3	*72*	*100*	*10*
4	*96*	*120*	*12*
5	*120*	*140*	*14*
6	*144*	*160*	*16*
7	*168*	*180*	*18*
8	*192*	*210*	*21*

9	*216*	*240*	*24*
10	*240*	*260*	*26*

18) calculate the amount of cell suspension required (C mL) for addition to the plating medium by dividing the total number of cells required (from Table 2) by the number of cells per mL of cell suspension (e.g. 7.5 x 106 cells.mL-1)
- e.g. C mL = 14x106 cells (for 5 plates) / 7.5x106 cells.mL-1 = 1.87 mL of cell suspension
Note: a resorufin calibration curve will be prepared with each assay. Include this plate in all calculations. For example, if 3 plates are needed for the assay, you will actually prepare 4 plates. The plate for the resorufin calibration curve should be processed the same way as the treatment plates

19) subtract C mL from the total volume of inoculated medium that is required = i.e. inoculated medium volume - C mL = adjusted volume of medium
- e.g. 160 mL vol. - 1.87 mL cell suspension = 158.13 mL medium

20) use a sterile 25 mL pipette or graduated cylinder to add the adjusted volume of medium into a sterile glass container and then the cell suspension (C mL)

21) mix the inoculated medium and use a sterile Eppendorf repeater pipette to quickly dispense 500 ③L portions to the wells of the culture cluster; avoid letting the cells settle out of suspension (i.e. mix cell suspension by tube inversion prior to filling the repeater tip). Refill the pipette tip for every 8 wells. Note: uneven distribution of cells across the multiwell plate has caused serious problems with the assay in the past. For added safety the cell suspension can be gently stirred in a sterile beaker for the duration of the well inoculation procedure. Stirring can be omitted if a repeating pipettor is used to re-suspend the cells in the beaker.

22) place the inoculated multi-well culture plates in the CO2 incubator at 37 oC . Incubate for 24 hours prior to treatment. This time can be increased if necessary, but for strict protocols, a 24 h plating period is recommended. Do not seal the plate.

Cell exposure:
(Day 2: 24 hours after plating)
Materials

- *culture of H4IIE cells grown in 48-well culture clusters (Costar, 3548)*
- *Nichiryo Model 800 repeater pipette with sterile glass tips*
- *sterile pasteur pipettes*
- *aspirator*
- *2,3,7,8-T4CDD standards*
- *dilution series of the "test" samples*

Concentration of Dioxin Standards:

Dose should be expressed as Molar units in the exposure system i.e. per well. Therefore, the 2,3,7,8-T4CDD standards should be labelled not only with the concentration (g.mL-1) in the amber vial, but also with the well dose (M).

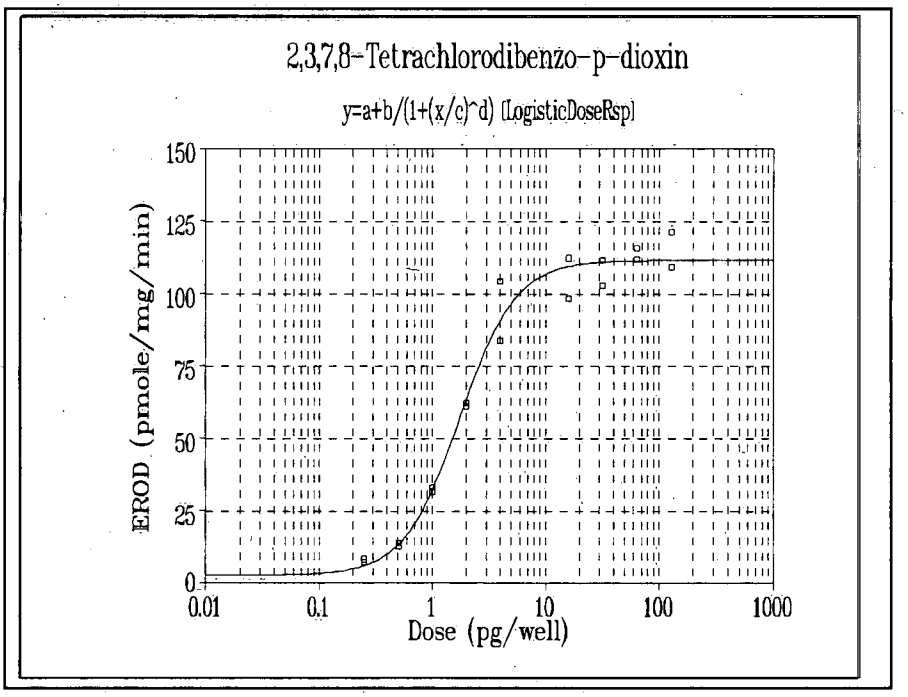
Dose is calculated as follows:
= [[Concentration of stock (g.L-1) x volume added to well (L)]/MW of compound (g.mole-1)]/volume of liquid in well (L)
eg [[0.0000128 g.L-1 x 0.0000025 L]/320 g.mole-1]/0.0005L = 200 pM

Table 3. Dioxin Standards.

Dilution	Concentration of Standard (ng.mL-1)	Mass per Well (pg.well-1)	Dose in Well (pM)
1	Blank	Blank	Blank
2	0.1	0.25	1.6
3	0.2	0.5	3.13
4	0.4	1	6.25
5	0.8	2	12.5
6	1.6	4	25
7	3.2	8	50
8	6.4	16	100
9	12.8	32	200
10	64	64	400
11	25.6	128	800

Ensure that the concentrations used for the standard curve cover both extremes of the dose response curve (see Figure 1). Concentrations that are either higher and/or lower than the ones listed above, can be used as required. Prepare the calibration standards as described in the Appendix.

Figure 1. Typical calibration curve for 2,3,7,8-tetrachlorodibenzo-p-dioxin.


2,3,7,8-Tetrachlorodibenzo-p-dioxin
y=a+b/(1+(x/c)^d) [LogisticDoseRsp]
EROD (pmole/mg/min)
150
125
100
75
50
25
0
0.01
0.1
1
10
100
1000
Dose (pg/well)

Sample dilution:
**typically make a 2-fold dilution series of test samples in 100% DMSO*
**if necessary a 1:4, 1:10 etc. fold dilution will extend the concentration range*

Plate layout:
Figure 2. Setup for a triplicate run of standards and samples

	1	2	3	4	5	6	7	8
A	Neg. Con.	Std. 1a	Std. 2a	Std. 3a	Std. 4a	Std. 5a	Std. 6a	Std. 7a
B	Neg. Con.	Std. 1b	Std. 2b	Std.3b	Std. 4b	Std. 5b	Std. 6b	Std. 7b
C	Neg. Con.	Std. 1c	Std. 2c	Std. 3c	Std. 4c	Std. 5c	Std. 6c	Std. 7c
D	S1a	S2a	S3a	S4a	S5a	S6a	S7a	S8a
E	S1b	S2b	S3b	S4b	S5b	S6b	S7b	S8b
F	S1c	S2c	S3c	S4c	S5c	S6c	S7c	S8c

Neg. Con.: negative control (100% DMSO); Std: standard; S: sample; 1,2,3 ...: concentration of standard, sample number, or sample dilution; a,b,c: replicates

Figure 3. Set up for duplicate run of standards and samples

	1	2	3	4	5	6	7	8
A	Neg. Con.	Std. 1a	Std. 2a	Std. 3a	Std. 4a	Std. 5a	Std. 6a	Std. 7a
B	Neg. Con.	Std. 1b	Std. 2b	Std.3b	Std. 4b	Std. 5b	Std. 6b	Std. 7b
C	S1a	S2a	S3a	S4a	S5a	S6a	S7a	S8a
D	S1b	S2b	S3b	S4b	S5b	S6b	S7b	S8b
E	S9a	S10a	S11a	S12a	S13a	S14a	S15a	S16a
F	S9b	S10b	S11b	S12b	S13b	S14b	S15b	S16b

Neg. Con.: negative control (100% DMSO); Std: standard; S: sample; 1,2,3 ...: concentration of standard, sample number, or sample dilution; a,b,c: replicates

Samples and standards should be prepared with 7 and 8 levels of dilution respectively.
Routinely prepare a 7 point dilution series for the standards.
For dilution series of samples that are replicated in triplicate, repeat the standard curve for every fifth sample, which is every 4th plate.
For individual samples that are tested in triplicate repeat the standard curve on every second plate.
For dilution series of samples that are replicated in duplicate, repeat the standard curve on every second plate.
For individual samples that are tested in duplicate, repeat the standard curve on every plate

Treatment procedure:

1) *warm the growth medium to 37oC*
2) *aspirate medium from the wells and add 500 @L of fresh medium per well. Note that some laboratories have eliminated this step since the exposure period is only for 48 h. A future experimental comparison may allow us to do likewise. The medium change is included for now.*

3) *use a positive displacement adjustable pipette with glass capillaries (plastic tips can adsorb hydrophobic chemicals and may interact with organic solvents) to add 2.5 ③L of treatment or control solution to each well; add the solution to the centre of the well; be careful not to dislodge cells from the well surface*
4) *swirl the plate between samples to mix the DMSO into solution. DMSO is toxic to cells upon contact, the swirling action helps dilute the DMSO so that few cells are affected*
5) *dose cells from low to high concentration using the same capillary pipette. Be sure to change the capillary pipette between samples. Note: 2.5 ③L dispensed into 500 ③L results in a 0.5% v/v DMSO concentration in the well. Caution is advised here since cells are sensitive to DMSO. Typically cells do not respond well to concentrations >1% DMSO (v/v)*
6) *return the dosed cells to the 37 oC incubator for a 48 h period. Note: the exposure period can be adjusted depending on the metabolic kinetics of the test chemical. Exposure to readily metabolized compounds can be shortened to 24 h.*

EROD assay:
(Day 3: 48 hours after dosing)
Materials

**repeater pipettes: 10 ③L, 250 ③L, and 500 ③L*
**adjustable pipettes: 100 - 1000 ③L, 10 - 100 ③L and tips*

Reagents

**HEPES Buffer at pH 7.8; N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (Sigma, H-7006). Dissolve 26.0 g HEPES sodium salt (MW 260.3) in 100 mL of distilled water (0.1M), adjust pH to 7.8.*

**Phosphate buffered saline at pH 7.5 is prepared in the laboratory and does not need to be sterilized. Store at 4 oC. Ingredients are:*

0.4 g of KCl
0.4 g of KH2PO4
16 g of NaCl
2.3 g of Na2HPO4

make up in 2 L distilled water and adjust to pH 7.5.

**Dicumarol (photosensitive): 3.3 mg mL-1 stock solution of dicumarol in DMSO. Dissolve 165 mg of dicumarol in 50 mL DMSO.*

7-Ethoxyresorufin (7-ER solution)
(Sigma, E-3763)

**stock solution: 40 ③g.mL-1 of ER (166 ③M) in Methanol or DMSO*
**read absorbance at 470 nm on spectrophotometer*
**adjust the absorbance to 2.70; 40 ③g.mL-1 of ER is an excess amount, so this adjustment is not critical*

** Working ER-Dicumarol reagent: ER stock solution is diluted 33x in HEPES (1.2 @g.mL-1; 5 @M). Dicumarol is diluted 333x in HEPES (1 @g.mL-1: 2.98 @M).*
Store at room temperature.
For 2 plates we shall need 96 wells x 150 @L of ER-Dicumarol reagent = 15 mL allowing for spillage.
A 33-fold dilution of ER in HEPES is 15 mL/33 = 0.455 mL of ER stock.
A 333-fold dilution of dicumarol in HEPES is 15 mL/333 = 0.045 mL of dicumarol stock.
Thus, add 14.5 mL of HEPES plus 0.455 mL of ER stock plus 0.045 mL of dicumarol to a tube and vortex mix. Mix multiples of those amounts in a beaker for larger experiments.

NADPH has been omitted from this version of the assay since it has been found to be non-essential. Live cells have their own supply of NADPH, plus it is unlikely that NADPH can traverse the membrane of live cells.

Cytofluor 2300 parameters for EROD assay

Figure 4. 7-ER Cytofluor Parameters.

Plate - Costar 48

	Wavelength	*Bandwidth*
Filter Ex - C	*530*	*25*
Filter Em - C	*590*	*35*

Sensitivity - 2 - 3 (adjust as necessary)

Note: the Cytofluor probe must be turned ON for more than 1 h before the assay. To do this set up a file such that multiple scans are run over an hour. Scans must run < 10 minutes apart so that the probe does not default to OFF. The light intensity will not change significantly after this warm-up period. Newer models may not require this precaution.

Enzyme assay procedure:
(48 well plates)
1) a. open Cytofluor file

b. pick file and then duplicate it
c. for each plate create a t0 and t10 file

2) *prewarm to 37 oC all solutions that will be added to cells*
3) *aspirate medium from each well; tilt plate, insert pipette tip at the edge of the well. Do not mar the cell surface. Add 100 ③L of PBS to each well to remove any residual inducer prior to the enzyme assay. Remove the PBS by aspiration or by inversion over a foil lined catch basin.*
4) *add 150 ③L of ER-Dicumarol reagent; dicumarol inhibits resorufin conjugation by diaphorase, and is needed in the H411E assay. Fish cells do not seem to require the addition of dicumarol[24].*
5) *start timer, agitate (on shaker) for 30 seconds*
6) *at 2 minutes place plate in Cytofluor*
7) *read plate: this is the t0 reading*
8) *incubate for 10 minutes on a fast shaker at 37 oC - the next plate can be started while this plate is incubating*
9) *save the scanned file and export as a CSV (comma separated value) file - this allows the file to be imported to a spreadsheet*
10) *open file (e.g. H1-10) for t10 reading*
11) *scan plate; this is the t10 reading*
12) *repeat for all subsequent plates*
13) *remove the assay fluid from the wells by aspiration or inversion*
14) *add 100 ③L of PBS to each well*
15) *remove PBS by aspiration or inversion*
16) *add 250 ③L of sterile distilled water to each well*
17) *wait 10 minutes for cells to dilate (cells appear round under low magnification)*
18) *place cells in -80 ®C freezer (or dry ice) until frozen and store until ready to do the protein assay*
19) *note: a resorufin calibration curve is run with each assay*

Resorufin standard curve
Materials

- *HEPES Buffer*
- *7-ER in HEPES Buffer*
- *Resorufin*
- *Eppendorf Repeater Pipette and tips: 10-, 250-, 500- ③L*

Resorufin standard stock solution

7-hydroxy-3H-phenoxazine-3-one
(Sigma, R-3257)

**dissolve 4.7 mg of resorufin in 100 mL of DMSO (or methanol) (200 ③M) = stock solution.*

At least 5 standards in the range of 1 to 500 nM (in the final reaction mixture) should be used to construct the resorufin standard curve. Resorufin degrades quickly (within weeks) at room temperature. Storage in the -70 oC freezer between standard curves is recommended. Prepare the top working stock (#1: 8000 nM) by adding 400 @L of stock solution (200 @M) to 9600 @L DMSO. Obtain 6 glass tubes, label #2 to #7 and add 5 mL of DMSO to each. Prepare 1 in 2 (1:1) serial dilutions of the resorufin stock #1 in the remaining 6 tubes. Store the calibration standards in amber vials at 4 oC.

Table 4. Resorufin Standard Concentrations

Vial	*Concentration (nM)*	*Final concentration in well (nM)*
1	*8,000*	*533*
2	*4,000*	*266.7*
3	*2,000*	*133.3*
4	*1,000*	*66.7*
5	*500*	*33.3*
6	*250*	*16.7*
7	*0*	*0*

Procedure:

1) *remove the medium and rinse the wells as described for the treatment plates*
2) *add 140 @L of ER-Dicumarol reagent to each well*
3) *add 10 @L of the appropriate resorufin standard to each well - use each column of the plate for a different concentration (see Figure 5)*
4) *scan plate using the same cytofluor parameters as the EROD assay (Figure 4)*
5) *remove the reaction mixture from the plate by inversion over a catch basin, or by aspiration*
6) *add 100 @L of PBS to each well. Remove the PBS by aspiration or inversion.*
7) *add 250 @L of sterile distilled water to each well. Allow the plate to stand for 5 minutes, then transfer to a -80oC freezer.*

Figure 5. Resorufin Standard Curve.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
A	*Neg. Con.*	*1a*	*2a*	*3a*	*4a*	*5a*	*6a*	*7a*
B	*Neg. Con.*	*1b*	*2b*	*3b*	*4b*	*5b*	*6b*	*7b*
C	*Neg. Con.*	*1c*	*2c*	*3c*	*4c*	*5c*	*6c*	*7c*
D	*Neg. Con.*	*1d*	*2d*	*3d*	*4d*	*5d*	*6d*	*7d*
E	*Neg. Con.*	*1e*	*2e*	*3e*	*4e*	*5e*	*6e*	*7e*
F	*Neg. Con.*	*1f*	*2f*	*3f*	*4f*	*5f*	*6f*	*7f*

Neg. Con.: Negative Control (100% DMSO)
1-7: refers to the dilution number
a-f: replicate

Protein assay [25]
It is preferable to run the protein assay on the same day as the EROD assay since Kennedy (personal communication 1997) observed changes in protein values after freezer storage.

Materials

**phosphate Buffer at pH 8.0; 7.49 g K2HPO4 and 0.925 g of KH2PO4 in 1L H20, adjust pH to 8.0*

**fluorescamine (MW 278.27 Aldrich; 30 mg per 100 mL acetonitrile; 1.1 mM). Because fluorescamine degrades quickly, this reagent must be freshly prepared for each assay.*

**acetonitrile is the carrier solvent for the fluorescamine.*

**distilled water*

**repeater pipettes: 250 ③L x 2; 500 ③L*

Preparation of calibration curve

BSA Standard (Bovine Serum Albumin, Bovine Fraction V (Sigma, A-9418) is used to generate the protein standard curve. Protein concentrations in each well should be between 10 and 200 ③g.mL-1 for interpolation of protein levels in individual wells.

**100 mg of BSA in 250 mL phosphate buffer at pH 8; prepared fresh*
**gives a 400 ③g mL-1 concentration*
= 200 ③g/0.5 mL
= 200 ③g/well
**make a 1:1 serial dilution series in 10 mL test tubes*

Dilution series:

**200 ③g/well*
**100 ③g/well*
**50 ③g/well*
**25 ③g/well*
**12.50 ③g/well*
** 6.25 ③g/well*
** 3.13 ③g/well*
** 0.00 ③g/well (phosphate buffer, only)*
**dilute 80 mg in 200 mL phosphate buffer (400 ③g mL-1 concentration)*

Procedure for calibration curve:

1) *add 250 ③L of distilled water to each well of a fresh plate*
2) *add 500 ③L of BSA standards in phosphate buffer to the appropriate wells - use each column of the plate for a different concentration (see Figure 6)*
3) *add 250 ③L of fluorescamine*
4) *wait 5 minutes ± about 20 seconds*
5) *take reading*

Figure 6. Setup for Protein Standard Curve.

	1	2	3	4	5	6	7	8
A	Neg. Con.	1a	2a	3a	4a	5a	6a	7a
B	Neg. Con.	1b	2b	3b	4b	5b	6b	7b
C	Neg. Con.	1c	2c	3c	4c	5c	6c	7c
D	Neg. Con.	1d	2d	3d	4d	5d	6d	7d
E	Neg. Con.	1e	2e	3e	4e	5e	6e	7e
F	Neg. Con.	1f	2f	3f	4f	5f	6f	7f

Neg. Con.: negative control (100% DMSO)
1-7: refers to the dilution number
a-f: replicate

Procedure for samples:

1) *change the cytofluor parameters (see Figure 7)*
2) *thaw cells in 37®C incubator*
3) *wipe any condensation from plate*
5) *add 500 ③L of phosphate buffer pH 8 - this optimizes the pH for the fluorescamine reaction*
6) *agitate on shaker for 30 seconds*
7) *start timer and add 250 ③L fluorescamine (30 mg/100 mL of acetonitrile) to each well 8) wait 5 minutes*
9) *scan the plate*

Table 5. Summary of protein assay components

Reagent	48-Well plate volumes (③L)	Concentration per well (reaction mixture)
distilled H2O	250	
Phosphate buffer	500	
Fluorescamine	250	0.2 ③M
Total volume	1,000	

Figure 7. Cytofluor Parameters for Protein Assay

Wavelength Bandwidth

Filter Ex - A *360 40*

Filter Em - A *460 40*

Sensitivity - 3 (adjust if necessary)

Data Analysis:
is described after the section on the PLHC-1 bioassay.

PLHC-1 Bioassay

Background
The introduction to this section is drawn directly from information provided by John A. Ryan of Dr. Hightower's Laboratory at the University of Connecticut.

History:
Hepatic tumours were induced by Schulz and Schulz, University of Connecticut, in Poeciliopsis lucida, a topminnow, in 1982 using multiple doses of 7, 12-dimethylbenz(a)anthracene[26,27]. One of these tumours, a differentiated hepatocellular carcinoma, was serially transplanted approximately 11 times by Dr. Mary Schulz. Tissue from this tumour was removed and dissociated in a trypsin-EDTA solution. The dissociated cells were pooled and cultured at room temperature in Eagle's MEM with 10% FBS. That cell culture was used to establish the PLHC-1 cell line.

Growth conditions:
PLHC-1 cells are normally grown at 30 oC in a humidified incubator under a 5% CO2 atmosphere. The cells are cultured in Eagle's minimum essential medium (MEM) with Earle's salts (Gibco-BRL) plus 5% (v/v) FBS at a pH of 7.2 - 7.4. Under those culture conditions there is no need to seal the culture flasks or multiwell clusters with Parafilmtm. So doing prevents the growth medium's buffering system from functioning correctly and can adversely affect cell growth. Hepes (25 mM) can be included in the medium if a CO2 incubator is not available.
PLHC-1 cells that are grown in 75 cm2 flasks are harvested by dissociation with Trypsin/EDTA as described in the present report. The PLHC-1 cells come off the bottom of the flask as small clumps. Attempting to break these clumps into single cells by repeated pipetting can greatly reduce viability. As PLHC-1 cells grow poorly at low dilutions, a split ratio of 1:8 or less is recommended.
The cells were received at NWRI as passage 92 cells. They were refrozen in liquid nitrogen at passage 94. Hahn et al. (1996) have shown that passage number affects the responsiveness of PLHC-1 cells to MFO inducers. The optimal passage number has yet to be established.

Morphology (Ryan personal communication, 1995):
PLHC-1 cells plated in plastic culture vessels retain some of the characteristic morphology of primary liver hepatocytes, forming cord like growth patterns with sinusoid-like openings between the rows of cells. The cells are epithelial, averaging 15 - 20 ③m across when spread on a surface and only 10 ③m when in suspension. Due to their small size and tight packing, they are able to achieve high densities (2x106 cells/cm2) at confluence in monolayer culture - that is 1.50x108 per 75 cm2 flask - far in excess of what is typically achieved in a culture of H411E cells. PLHC-1 cells grown on plastic substrates show a less structured cytosolic organization than liver parenchyma cells but they still retain several expected differentiated characteristics including abundant deposits of glycogen, tight junctions near the apical surface that are exposed to the culture medium, and basolateral interdigitations.

Growth rate

When heavily confluent cultures are split 1:4 they again become confluent within 48 hours although continued growth is required to achieve the original cell density. PLHC-1 cells are not contact inhibited. Long term cultures can form ridges and pile up. Average doubling time at 30 oC is 39.4 hours.

Thus, the PLHC-1 cell line has some important characteristics that distinguish it from other fish-cell lines. Those characteristics will influence protocols for PLHC-1 based bioassays.

Cell plating
(Day 1)
Materials

- *laminar flow hood*
- *48-well culture clusters (Costar CS003548)*
- *Trypsin (0.05%)-EDTA (0.5 mM) (Gibco-BRL 25300-047)*
- *Eagle's Minimum Essential Medium (MEM) with Earle's Salts and L-glutamine (GibcoBRL 11095-080)*
- *Penicillin (10,000 units.mL-1)-Streptomycin (10,000 ③g.mL-1)(GibcoBRL 15140-031)*
- *Non-essential Amino Acids (GibcoBRL 320-1140PG) (100x)*
- *Fetal Bovine Serum (FBS, GibcoBRL 16000-010)*
- *HEPES buffer (1.25 M at pH 7.0, Sigma 7-9 Cat. No. T-1378)*
- *fungizone (Gibco BRL 15295-017)*
- *sterile pipettes: 10, 25 or 50 mL*
- *sterilized graduated cylinders (10-100 mL)*
- *pipette-aid*
- *sterile 100-500 mL glass containers (Erlenmeyer flasks or glass bottles)*
- *aspirator*
- *haemocytometer*
- *microscope (10x10 magnification)*
- *15 and 50 mL sterile disposable centrifuge tubes (Falcon, Costar, or Corning)*
- *repeater pipette*
- *sterile 250 ③L tips*
- *sterile pasteur pipettes and bulbs*
- *adjustable pipettes (10-1000 ③L capacity) with sterile tips*
- *200-500 mL autoclaved glass containers (Erlenmyer flasks, glass bottles)*
- *1.2 mL cryovials*

Media preparation
Stock solutions:
HEPES (MW 260.3, pH 7) at 1.25 M is prepared as follows, filter sterilized, and stored in a sterile container at 4 oC: dissolve 32.5 g HEPES in 100 mL deionized H2O; adjust to pH 7.0. To be used when a CO2 incubator is not available. 10 mL of 1.25 M Hepes is added to 450 mL MEM with Earl's salts.

Table 6. Ingredients for PLHC-1 medium

Ingredients	*Volume Needed (mL)*
Minimum Essential Medium (MEM) with Earle's salts	*460*
Non-essential amino acids (100x)	*5*
Penicillin-Streptomycin	*5*
Fungizone	*5*
*Fetal Bovine Serum (FBS)**	*25*

** 10% (v/v) FBS can be used during routine culturing to boost proliferation. 5% FBS should be used in the bioassay.*
Note: FBS is stored at - 20oC. It can be bought in 100 or 500 mL quantities. Larger volumes (less expensive) can be distributed into sterile containers (50 mL centrifuge tubes recommended) in appropriate amounts.
MEM, non-essential amino acids is stored at 4 oC.
Aliquots (5 mL) of the antibiotic solutions are stored at -20oC.

Procedure:

1) *thaw trypsin*
2) *warm trypsin, medium, and PBS to 30o C in a water bath*
3) *aspirate medium from the culture flask(s)*
4) *gently rinse cells with 5 mL of PBS;*
5) *aspirate the PBS*
6) *add 2 mL of trypsin reagent to each culture flask; the number of flasks depends on the size of the experiment*
7) *rock flask periodically to facilitate trypsin reaction*
8) *wait about 5-10 minutes for the cells to lift*
9) *stop the trypsin reaction by the addition of 5 mL of MEM*
10) *pipette the resulting cell suspension into 10 mL centrifuge tube*
11) *centrifuge for 5 minutes (500 g)*
12) *aspirate most of the supernatant (leave about 1 mL)*
13) *mix pellet and supernatant*
14) *top up solution to 5 mL*
15) *count the cells in solution as previously described*
16) *calculate the number of cells per mL and the total number of cells as described for the H411E cells*
17) *determine the amount of medium required by multiplying the number of individual wells per culture cluster (typically 48) to be used by 0.5 mL.well-1 by the number of plates to be used. Always round up to allow for spills. The rounded-up volume is referred to as the working volume.*
    = # wells.plate-1 x 0.5 mL.well-1 x 4 plates = 96 mL; round up to 110 mL see Table 7
18) *calculate the number of cells required by multiplying the working volume by 8 x 105 cells.mL-1, as indicated in Table 7. That means that 4.0 x 105 cells will be added to each well. Using similar assay conditions Hahn et al. (1996) established a relationship between cells/well and resorufin formation. There was a more than 10 fold increase in the assay signal (not normalized for protein level) in response to 2,3,7,8-T4CDF when the number of cells per well was increased from 1 x 105 per well to 5 x 105 cells per well.*

Hahn standardized on an inoculum density of 4 x 105 cells per well in 0.5 mL of medium. We have yet to experiment with the effect of inoculum size on assay performance. In the present report we use an inoculum size of 4 x 105 cells per mL based on the published data of Hahn et al.[18].

Table 7. Number of cells needed

Number of Plates	*Required volume (mL)*	*A-Working volume (mL)*	*B-Cells per mL*	*AxB = Cells required*
1	*24*	*30*	*8.0 x 105*	*2.4 x 107*
2	*48*	*60*	*8.0 x 105*	*4.8 x 107*
3	*72*	*80*	*8.0 x 105*	*6.4 x 107*
4	*96*	*110*	*8.0 x 105*	*8.8 x 107*
5	*120*	*130*	*8.0 x 105*	*1.04 x 108*
6	*144*	*150*	*8.0 x 105*	*1.2 x 108*

19) calculate the volume of cell suspension required for the plating suspension by dividing the total number of cells required (Table 7) by the total cell number and multiplying by the volume of the total cell suspension:

= (cells required / number of cells harvested) x suspension volume
e.g. (8.8 x 107 cells (for 4 plates) / 1 x 108 cells) x 5 mL = 4.4 mL of cell suspension required

20) determine the amount of medium needed by subtracting the amount of suspension required from the total volume of inoculated medium that will be needed to seed the multi-well plates
= medium volume required - cell suspension required
= medium volume required (adjusted)
e.g. = 110 mL - 4.4 mL = 105.6 mL of medium required + 4.4 mL of cell suspension

21) add the required volume of medium (adjusted) with a sterile pipette or graduated cylinder into a sterile glass container, then add the cell suspension to the container

22) distribute cells in 500 ③L portions (quickly to avoid adherence to glass surface or settling) into the 48-well plate using a sterile Eppendorf repeater pipette

23) label plates (date, passage #, cell/well, etc) and place in CO2 incubator

23a) do not seal the plates

24) the plates will remain in the incubator for 24 hours prior to treatment with the standards and test compounds. This time can be increased if necessary, but for strict protocols, a 24 h period is recommended.

Cell exposure

Materials

**aspirator*
**culture medium*
**repeater pipette and 250 ③L tips*
**adjustable positive displacement pipette with glass capillary pipettes*
**2,3,7,8-T4CDD standards*

*test samples in dilutions.

Dioxin standards for PLHC-1 bioassay
Use the protocol that has been provided for the H411E based bioassay

Sample Dilution

1) *typically make a 2 to 5-fold dilution series of test samples in 100% DMSO. Note: DMSO is the usual diluent for cell dosing, at levels of 2.5 ③L per well. We have also had success using isooctane as a diluent at levels of up to 5 ③L per 500 ③L well. Isooctane as a diluent has the advantage that the left over isooctane solutions can chemically analyzed after the cell dosing procedure.*
2) *use either: one half or the whole plate for each sample depending on the concentration at which a response is expected. If necessary use a 1:4, 1:10 etc. fold dilution to extend the concentration range. See the H4IIE procedure for further guidance.*

Procedure:

1) *warm medium to 30oC*
2) *aspirate medium from the wells and immediately add 500 ③L fresh medium to each well*
3) *add 2.5 ③L of treatment or control solution to each well - use the positive displacement pipette with glass capillaries (plastic tips can adsorb hydrophobic chemicals)*
4) *swirl the plate between samples to mix the DMSO (or isooctane, see above note) into solution. DMSO is toxic to cells upon contact, but this swirling should dilute the DMSO so that few cells are affected*
5) *dose cells from low to high concentration using the same capillary pipette. Be sure to change capillary pipettes between samples.*
   Note: 2.5 ③L dispensed into 500 ③L results in a 0.5% (v/v) DMSO concentration in the well. Be careful with this ratio since cells are very sensitive to DMSO. Typically cells do not respond well to >1% DMSO (v/v)
6) *return the dosed microwell plates to the 30 oC incubator.*

EROD assay
Instruments and accessories:

- *sterile 250 ③L pipette tips*
- *repeater pipette*
- *100, 250, and 500 ③L pipette tips*
- *adjustable pipette 100-1000 ③L, 10-100 ③L and tips*

Assay reagents
1. Tris-NaCl buffer (TN) for EROD assay solution, 0.05 M Tris (Sigma 7-9), 0.1 M NaCl, pH 7.8. Store at 4oC.

6.05 g of Tris (MW = 121.1)
5.84 g of NaCl (MW = 58.4)

is added to 1 litre of distilled water and the pH is adjusted to 7.8

2. 7-Ethoxyresorufin (7-ER, MW = 241.2, Sigma) is dissolved in methanol to give a concentration of 166 ③M (40 ③g.mL-1). This stock is diluted 82.5x in TN buffer which results in a 2 ③M solution. Store at room temperature.

3. Resorufin in DMSO (MW = 235.2, Sigma) is used to prepare standard curves. At least 5 standard solutions in the range of 0.58 - 500 nM (in the final reaction mixture) should be used to construct a resorufin standard curve. Resorufin degrades quickly (within weeks) at room temperature. Storage at -70 oC is recommended.

4. Cold methanol is used as a solvent for resorufin and 7-ER
5. Phosphate buffered saline at pH 7.5 is prepared in the laboratory and does NOT need to be sterilized. Store at 4 oC. Ingredients are:

0.4 g of KCl
0.4 g of KH2PO4
16 g of NaCl
2.3 g of Na2HPO4

make up in 2 L distilled water and adjust to pH 7.5.

Assay procedure:
Cytofluor plate reader: set up as described for the H411E assay; adjust instrument sensitivity as required.

Before the EROD assay is started prepare the 7-ethoxyresorufin reagent. 150 ③L per well of this reagent will be required. Prepare excess. Pre-warm all solutions to 30 oC.

The EROD assay is run as follows (use Eppendorf repeater pipette):

1) *aspirate medium from each well, tilting the plate and inserting the pipette tip at the edge of the well. Do not mar the cell surface.*
2) *add 100 ③L of PBS to each well*
3) *aspirate each well*
4) *immediately add 150 ③L of the 7-ER reagent*
5) *start timer. Let reaction occur for approximately 2 minutes*
6) *stop timer. Insert plate into scanner, start timer and scan plate. This is the t=0 reading*
7) *reset and restart the timer. Place the plate on a fast shaker at 30 oC*
8) *after 10 minutes, insert plate into scanner and scan. This is the t=10 reading*
9) *upon completion of the t=10 scan, aspirate the reaction mixture*
10) *add 100 ③L of PBS to each well*
11) *aspirate each well*
12) *add 250 ③L of sterile distilled H2O*
13) *after 10 minutes or more, place plates into an -80oC freezer. The plates can remain frozen until it is time to run the protein assay. Although, it is recommended to run the protein and EROD assays on the same day.*

Table 8. Summary of EROD reaction mixture components

Reagent	*48-Well plate volumes*	*Concentration per well (reaction mixture)*
7-ER in TN Buffer	*150 mL*	*2 ③M*
total volume	*150 ③L*	

A standard curve with resorufin is performed with every assay using the following modification of the method described for the H411E EROD assay.

Resorufin standard curve
Materials

- *HEPES Buffer*
- *7-ER in HEPES Buffer*
- *Resorufin*
- *Eppendorf Repeater Pipette and tips: 10-, 250-, 500- ③L*

Resorufin standard stock solution

7-hydroxy-3H-phenoxazine-3-one
(Sigma, R-3257)

- *dissolve 4.7 mg of resorufin in 100 mL of DMSO (or methanol) (200 ③M) = stock solution.*

At least 5 standards in the range of 1 to 425 nM (in the final reaction mixture) should be used to construct the resorufin standard curve. Resorufin degrades quickly (within weeks) at room temperature. Storage in the -70 oC freezer between standard curves is recommended. Prepare the top working stock (#1: 6378 nM) by adding 160 ③L of stock solution (200 ③M) to 9840 ③L DMSO. Obtain 6 glass vials, label #2 to #7 and add 60 ③L of DMSO to each. Prepare 1 in 3 (1:2) serial dilutions of the resorufin stock #1 in the remaining 6 vials. Store the calibration standards in amber vials at 4 oC.

Procedure:

1) *remove the medium and rinse the wells as described for the treatment plates*
2) *add 140 ③L of ER-Dicumarol reagent to each well*
3) *add 10 ③L of the appropriate resorufin standard to each well - use each column of the plate for a different concentration (see Figure 5)*
4) *scan plate using the same cytofluor parameters as the EROD assay (Figure 4)*
5) *remove the reaction mixture from the plate by inversion over a catch basin, or by aspiration*
6) *add 100 ③L of PBS to each well. Remove the PBS by aspiration or inversion.*
7) *add 250 ③L of sterile distilled water to each well. Allow the plate to stand for 5 minutes, then transfer to a -80oC freezer.*

Table 4. Resorufin Standard Concentrations

Vial	*Concentration (nM)*	*Final concentration in well (nM)*

1	6,377.55	425.17
2	2,125.9	141.22
3	708.6	47.24
4	236.2	15.75
5	78.7	5.75
6	26.24	1.75
7	8.75	0.58

Figure 5. Resorufin Standard Curve.

	1	2	3	4	5	6	7	8
A	Neg. Con.	1a	2a	3a	4a	5a	6a	7a
B	Neg. Con.	1b	2b	3b	4b	5b	6b	7b
C	Neg. Con.	1c	2c	3c	4c	5c	6c	7c
D	Neg. Con.	1d	2d	3d	4d	5d	6d	7d
E	Neg. Con.	1e	2e	3e	4e	5e	6e	7e
F	Neg. Con.	1f	2f	3f	4f	5f	6f	7f

Neg. Con.: Negative Control (100% DMSO)
1-7: refers to the dilution number
a-f: replicate

Protein assay

The slope of the protein standard curve generally ranges from 18-28 at sensitivity 3 using the Cytoflour 2300. Typically protein levels should range from 80-100 @g.mL-1 for wells inoculated with 4 x 105 cells per well.

The protein assay is the same as described for the H411E assay.

It is preferable to run the protein assay on the same day as the EROD assay since Kennedy (personal communication 1997) observed changes in protein values after freezer storage.

Materials

****phosphate Buffer at pH 8.0; 7.49 g K2HPO4 and 0.925 g of KH2PO4 in 1L H20, adjust pH to 8.0***

****fluorescamine (MW 278.27 Aldrich; 30 mg per 100 mL acetonitrile; 1:1 mM). Because fluorescamine degrades quickly, this reagent must be freshly prepared for each assay.***

****acetonitrile is the carrier solvent for the fluorescamine.***

****distilled water***

****repeater pipettes: 250 @L x 2; 500 @L***

Preparation of calibration curve
BSA Standard (Bovine Serum Albumin, Bovine Fraction V (Sigma, A-9418) is used to generate the protein standard curve. Protein concentrations in each well should be between 10 and 200 ③g.mL-1 for interpolation of protein levels in individual wells.

** 100 mg of BSA in 250 mL phosphate buffer at pH 8; prepared fresh*
** gives a 400 ③g mL-1 concentration*
= 200 ③g/0.5 mL
= 200 ③g/well
** make a 1:1 serial dilution series in 10 mL test tubes*

Dilution series

** 200 ③g/well*
** 100 ③g/well*
** 50 ③g/well*
** 25 ③g/well*
** 12.50 ③g/well*
** 6.25 ③g/well*
** 3.13 ③g/well*
** 0.00 ③g/well (phosphate buffer, only)*
** dilute 80 mg in 200 mL phosphate buffer (400 ③g mL-1 concentration)*

Procedure for calibration curve:

1) *add 250 ③L of distilled water to each well of a fresh plate*
2) *add 500 ③L of BSA standards in phosphate buffer to the appropriate wells - use each column of the plate for a different concentration (see Figure 6)*
3) *add 250 ③L of fluorescamine*
4) *wait 5 minutes ± about 20 seconds*
5) *take reading*

Figure 6. Setup for Protein Standard Curve.

	1	2	3	4	5	6	7	8
A	Neg. Con.	1a	2a	3a	4a	5a	6a	7a
B	Neg. Con.	1b	2b	3b	4b	5b	6b	7b
C	Neg. Con.	1c	2c	3c	4c	5c	6c	7c
D	Neg. Con.	1d	2d	3d	4d	5d	6d	7d
E	Neg. Con.	1e	2e	3e	4e	5e	6e	7e
F	Neg. Con.	1f	2f	3f	4f	5f	6f	7f

Neg. Con.: negative control (100% DMSO)
1-7: refers to the dilution number
a-f: replicate

Procedure for samples:

1) *change the cytofluor parameters (see Figure 7)*
2) *thaw cells in 37®C incubator*
3) *wipe any condensation from plate*
5) *add 500 ③L of phosphate buffer pH 8 - this optimizes the pH for the fluorescamine reaction*
6) *agitate on shaker for 30 seconds*
7) *start timer and add 250 ③L fluorescamine (30 mg/100 mL of acetonitrile) to each well 8) wait 5 minutes*
9) *scan the plate*

Table 5. Summary of protein assay components

Reagent	*48-Well plate volumes (③L)*	*Concentration per well (reaction mixture)*
distilled H2O	*250*	
Phosphate buffer	*500*	
Fluorescamine	*250*	*0.2 ③M*
Total volume	*1,000*	

Figure 7. Cytofluor Parameters for Protein Assay

	Wavelength	*Bandwidth*
Filter Ex - A	*360*	*40*
Filter Em - A	*460*	*40*

Sensitivity - 3 (adjust if necessary)

Data Analysis
Calculations:
Specific EROD activity can be calculated using the following equations:
EROD Activity = (pmoles resorufin)/(mg Protein x Reaction time)

EROD Activity = [Average EROD Fluorescent units / Slope of Resorufin Standard Curve (pmol)] / [(Protein Fluorescence / slope of protein curve (③g)) x 1 mg/ 1000 x Reaction time (min)] = pmol/mg/min

Adjusted EROD Activity = EROD Activity (treatments) - EROD Activity (carrier solvent)

Data processing:
Exporting Cytofluor Data Files: export as a CSV file. See Cytofluor manual or on line help.

Importing Files (Quattro Pro): import files into spreadsheet. Get directions from the on-line help or the user's manual.

The following procedure is used at NWRI and is detailed for the benefit of users at that site:

1) *import cytofluor files to Quattro Pro, using the Tools option*
   **Tools --> Import --> Only comma --> Directory --> (filename.csv)*
   **position the cursor at spreadsheet cell: A5 and import the t0 file*
2) *move cursor to C5 (cell will contain: "NAME", see figure 10, here "NAME" is in italics) and import the file for t10 (see Figure 10) - this will over write the data (on the screen) from the previous file (this data is not required for the calculations)*
3) *move cursor to E5 and import protein FU (florescence) file*
   - again this will over write the previous data

Figure 8. Typical .csv file imported to Quattro Pro.

FILE REF, ,NAME, , ,ASSAY, , , LAB
040895-001,,1a,,,TCDD - 10 dil,,,August 4, 1995
COMMENTS:-,,
,
SET,1,1,2,2,3,3,4,4,
FILTER, EX, EM, EX, EM, EX, EM, EX, EM,
CENTRE,530,590,
WIDTH,25,35,
POSN, C, C,
SENS.,3,3,

PLATE:-,Costar 48,,,COLS:-,8,ROWS:-,6,,
PASSES,1,PASS CYCLE TIME,,0,TOTAL TIME,,24

PASS:-,PASS 1, PASS 1, PASS 1, PASS 1,
SET:-,SET 1,SET 2,SET 3,SET 4,
A1,260,,,,
A2,280,,,,
A3,281,,,,
A4,280,,,,
A5,286,,,,
A6,283,,,,
A7,289,,,,
A8,285,,,,
B1,266,,,,

B2,266,,,,
etc.

4) *go to the bottom of the spreadsheet (column A) to import data from next cytofluor plate (repeat for all plates)*
5) *return to row "5" of the spreadsheet and import the data for the protein standard curve (import into a section of the spreadsheet that is not occupied)*
6) *move to another column (i.e. in row 5) and import the data for the resorufin standard curve*
7) *additional data will be imported with each of the above file, delete all but the column containing the concentration and the fluorescence (delete data in "bold", in figure 10)*
8) *set up the spreadsheet following the template in Figure 11 and Table 6*
9) *calculate the slope of the protein and resorufin standard curves using the linear regression subroutine in Quattro Pro - these will be necessary for columns "D" and "G" of the spreadsheet respectively (again, see figure 11)*
10) *sort individual plates (samples) using concentration as the primary sort key in Quattro Pro (Database --> Sort)*
11) *save the concentration and EROD values for every sample in individual files (Print --> Block [highlight concentration and EROD] --> Destination --> File [filename.prn] --> Spreadsheet Print --> Quit)*

Figure 9. Sample Spreadsheet1.

	A2	B	C	D	E	F	G	H
1	Well	T0 (FU)	T10 (FU)	T10 - T0 (FU)	Protein (FU)	Total Protein @g/well	Dose pM	EROD pmol/mg/ min
2								
3	...5	...	...	...	...	...	...	...
15	B2	266	279	13	2,292	99.82	0	0.48
16	B3	287	439	152	2,286	99.54	1.6	5.64
17	B4	276	487	211	2,185	94.75	3.13	8.23
18	B5	315	1,031	716	2,299	100.15	6.25	26.4
19	B6	315	3,071	1,155	2,072	89.38	12.5	47.7
20	...	...	...	...	...	...	...	...

1spreadsheet calculations (from cytofluor data) for microtitre plate wells "B2" to "B6"
2shaded regions are the rows and column of the spreadsheet
5ellipses represent rest of spreadsheet

Table 9. Calculations and Explanations of Spreadsheet1

Column	Title	Formulas and Explanations
A	Well	- the row and column of the micro titre plate

B	*T0*	*- the cytofluor reading at time0 in Fluorescence Units*
C	*T10*	*- the cytofluor reading at time10 in Fluorescence Units*
D	*T10 - T0*	*- the difference in reading between time10 and time0* *- Formula: +D15 - C15*
E	*Protein*	*- the cytofluor reading of the protein assay in Fluorescence Units*
F	*Total Protein*	*- total amount of protein (③g protein/well)* *- Formula: (E15 - protein blank2)/slope of protein std. curve3*
G	*Dose*	*- concentration of sample or standard in well (in pg/well)*
H	*EROD*	*- EROD activity (in pmoles resorufin/mg protein/min)* *- Formula: +D15/(RES. STD.4)/10/F15 * 1000* *- D15 - total amount of fluorescence* *- RES. STD. - resorufin standard curve* *- 10 - reaction time of 10 minutes* *- F15 - total protein* *- 1000 - converts from ③g to mg of protein*

1formulas in table refer to data from row 15 of Figure 9
2protein blank (from protein standard curve blanks)
3protein standard curve (slope, calculated in spreadsheet)
4RES. STD. - resorufin standard curve (slope, calculated in spreadsheet)

Table curve calculations

The final assay curve can be plotted in Table Curvetm, Jandel Scientific. The logistic dose response protocol gives good fits and allows interpolation of EC50 values.

Appendix
2,3,7,8-T4CDD
2,3,7,8-Tetrachlordibenzo-p-dioxin
(Cambridge Isotope Laboratories, ED-922)

1) start with 1.2 mL vial of 2,3,7,8-T4CDD at 50 ③g mL-1 nonane
2) transfer 1 mL into iso-octane
- 2x dilution is 25 ③g mL-1 concentration in (do all dilutions in 2mL volumetric flasks)
3) Working Stock
- transfer 8 ③L (250x dil.) from step 2 in isooctane (100,000 pg mL-1)
4) Lowest Concentration
- transfer 2 ③L (1000x dilution) from step 3 (working stock) into DMSO

- 100 pg mL-1 concentration (0.25 pg/well)

5) Sixth Highest Concentration

- transfer 4 ③L (500x dilution) from step 3 in DMSO

- 200 pg mL-1 concentration (0.50 pg/well)

6) Fifth Highest Concentration

- transfer 8 ③L (250x dilution) from step 3 in DMSO

- 400 pg mL-1 concentration (1.0 pg/well)

7) Fourth Highest Concentration

- transfer 16 ③L (125x dilution) from step 3 in DMSO

- 800 pg mL-1 concentration (2.0 pg/well)

8) Third Highest Concentration

- transfer 32 ③L (62.50x dilution) from step 3 in DMSO

- 1600 pg mL-1 concentration (4.0 pg/well)

9) Second Highest Concentration

- transfer 64 ③L (31.25x dilution) from step 3 in DMSO

- 3200 pg mL-1 concentration (8.0 pg/well)

10) Highest Concentration

- transfer 128 ③L (15.63x dilution) from step 3 in DMSO

- 6400 pg mL-1 concentration (16.0 pg/well)

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