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ARBOR ASSAYS™
Interactive Assay Solutions™

MULTI SPECIES

DetectX®

P450 Demethylating
Fluorescent Activity Kit

2 Plate Kit Catalog Number K011-F1

Sample Types Validated:

Demethylating P450 systems:
liver microsomes or cerosomes such as
Cyp P450 3A4, 2B4 and 2D6

Covered under US Patent numbers 8,173,386 & 8,765,396

Please read this insert completely prior to using the product.
For research use only. Not for use in diagnostic procedures.

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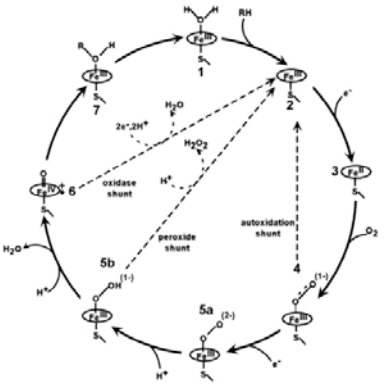
BACKGROUND

The cytochromes P450 (P450s) are a superfamily of heme containing enzymes that display tremendous diversity with regard to substrate specificity and catalytic activity[1,2]. P450s use a plethora of both exogenous and endogenous compounds as substrates in enzymatic reactions. Usually they form part of multicomponent electron transfer reactions (see figure). Catalysis by the eukaryotic P450 enzymes involves a multistep reaction cycle that includes two steps in which electron transfer is accomplished from a redox partner. The diflavin protein, NADPH cytochrome P450 reductase (reductase) contains both FAD and FMN and can transfer both electrons needed for the catalytic cycle[3]. In some P450 reactions, the second electron of the reaction cycle also can be delivered by cytochrome b5[4]. The P450 enzymes and cofactors of the mammalian drug-metabolizing system are embedded in the membrane of the endoplasmic reticulum[5]. The P450s play a crucial role in the development of new drug entities as drug-drug interactions commonly arise from the inhibition of cytochrome P450 activities.



Lipid plays an important role in the reconstitution of P450-dependent activities after protein purification[6]. Most in vitro studies for the reconstitution of P450 activities use dilaurylphosphatidylcholine (DLPC) as the lipid component. The reconstitution of enzymatic activity involves a concentrated incubation of P450, its redox partners (NADPH and reductase), and lipid followed by dilution into the final assay components. The reported preincubation conditions vary significantly[7].

1. Hollenberg, PF, “Mechanisms of Cytochrome P450 and peroxidase-catalyzed xenobiotic metabolism”, 1992, FASEB J 6(2):686-694.
2. Guengerich FP, “Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity” 2001, Chem Res Toxicol 14:611–650.
3. White RE and Coon MJ, “Oxygen activation by cytochrome P-450”. 1980, Ann Rev Biochem 49:315–356.
4. Schenkman JB and Jansson I, “The many roles of cytochrome B5”, 2003, Pharmacol Ther. 97:139–152.
5. Ortiz-De Montellano PR, “Oxygen activation and reactivity”, in Cytochrome P450: Structure, Mechanism and Biochemistry 1995, (Ortiz-De Montellano PR ed.) pp 245–303, Plenum Press, New York.
6. Lu AY, Strobel HW, and Coon MJ, “Hydroxylation of benzphetamine and other drugs by a solubilized form of cytochrome P-450 from liver microsomes: lipid requirement for drug demethylation”, 1969, Biochem Biophys Res Comm 36:545–551.
7. Causey KM, Eyer CS, and Backes WL, “Dual role of phospholipid in the reconstitution of cytochrome P-450 LM2-dependent activities”, 1990, Mol Pharmacol 38:134–142.

ASSAY PRINCIPLE

The DetectX® P450 Activity kit is designed to quantitatively measure the enzymatic activity of formaldehyde-producing enzymes such as Cytochrome P450s. The kit is unique in that the fluorescent substrate is not involved in the multicomponent P450 reaction, but measures the product of the demethylation, formaldehyde. No separation or washing is required. The kit has been validated for several P450 systems and should work with any biological system that is producing formaldehyde as a product of demethylation.

The kit provides an optimized buffer for P450, lyophilized vials of the cofactor NADPH for the reaction, a stable formaldehyde standard, the Formaldehyde Detection Reagent (FDR) and two 96 well plates for detecting the generated fluorescent signal. The end user will have to provide the microsomal, baculosome system or the recombinant P450, reductase and cytochrome b5 system and any cofactors, etc. necessary for activity, along with any candidate drugs, inhibitors or activators being tested. The reaction should be carried out in our supplied buffer or a similar PBS based buffer system.

Following the P450 NADPH-induced reaction, the generation of formaldehyde can be stopped by addition of a suitable inhibitor, or the supplied stop solution of acetic acid. The FDR is then added to all the wells. If calibration to formaldehyde is needed (for cross lab comparisons) then a formaldehyde standard curve generated from the supplied standard should be run.

After a short incubation at 37°C for 30 minutes, the fluorescent product is read at 510 nm in a fluorescent plate reader with excitation at 450 nm. The P450 activity is determined based upon formaldehyde production. We have provided two 96 well plates for measurement but this assay is adaptable for higher density plate formats. If substituting their own plates, the end user should ensure that their black HTS plate is suitable for use with these reagents prior to running samples.

SUPPLIED COMPONENTS

Black Half Area 96 Well Plate
See: www.ArborAssays.com/resources/#general-info for plate dimensions.
2 plates Catalog Number X037-2EA

Assay Buffer
A 100 mM potassium phosphate buffer at pH 7.4 containing 0.005% gentamicin.
60 mL Catalog Number X047-60ML

NADPH lyophilized
Reduced β-nicotinamide adenine dinucleotide 2'-phosphate (NADPH) freeze dried with stabilizers and stored in ziploc pouch with desiccant.
2 vials Catalog Number X045-1EA

Stop Solution
A 1M solution of Acetic Acid in water. **CAUTION: Acid solution.**
1 mL Catalog Number X046-1ML

Formaldehyde Standard
2,000 µM formaldehyde solution in deionized water. Outer container has formaldehyde absorbing pad. The standard is stable if kept tightly sealed. **KEEP TIGHTLY SEALED.**
500 µL Catalog Number C001-500UL

DetectX® Formaldehyde Reagent
Special formulation of reagents to detect formaldehyde in solution. Contains 0.09% sodium azide as a preservative.
5 mL Catalog Number C002-5ML

Plate Sealers
2 Each Catalog Number X002-1EA

STORAGE INSTRUCTIONS

All components of this kit should be stored at 4°C until the expiration date of the kit.

OTHER MATERIALS REQUIRED

Incubator capable of accurately maintaining 37°C.

P450 systems. Microsome, Cerosome, baculosome or supersome P450 systems, or recombinant P450, NADPH/P450 oxidoreductase and cytochrome b5 and Dilaurylphosphatidylcholine (DLPC) as the lipid used for reconstitution.

Repeater pipet with disposable tips capable of dispensing 25 µL.

Fluorescence 96 well plate reader capable of reading fluorescent emission at 510 nm, with excitation at 450 nm. Set plate parameters for a 96-well Corning Costar 3694 plate. See: www.ArborAssays.com/resources/#general-info for plate dimension data.

The sensitivity of fluorescent assays is dependant on the capabilities of the plate reader. If your plate reader has adjustable gain you can modify the signals obtained from the assay by increasing or decreasing the gain settings, by changing the aperture settings for monochromator based readers, or by changing the band pass width of the emission and/or excitation filters on some readers. Please review the plate reader manual for details.

Signals expressed in this insert are Relative Fluorescent Units (RFU) and were obtained on our plate readers. The RFU numbers you obtain may be different from these, but the assay results should be similar.

Software for converting raw relative fluorescent unit (FLU) readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

PRECAUTIONS

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

Formaldehyde is a toxic, volatile, reactive chemical that can form adducts with proteins and nucleic acids. It reacts with oxygen to form formic acid and so should be kept sealed and only used in well-ventilated laboratories. For disposal, we suggest discarding all excess standards and samples in a 10% aqueous solution of sodium bisulfite, such as Sigma catalog number 13438.

Some of the components of this kit contain sodium azide as a preservative, which may react with lead or copper plumbing to form potentially explosive complexes. When disposing of reagents always flush with large volumes of water to prevent azide build-up.

SAMPLE TYPES

P450 enzyme systems diluted in the supplied Assay Buffer provided or a typical 0.1M phosphate buffer at pH 7.4 are compatible with this assay.

P450 DEMETHYLATING REACTION CONDITIONS

We have ensured the DetectX® P450 Activity Assay detects the activity of the 2B4, 2D6 and 3A4 P450 systems. Below we have listed the conditions we used in validating this fluorescent detection system and the ability to quantitate the formaldehyde produced by the Cyp 2B4 P450 enzymatic reaction.

Typical Cyp 2B4 Enzyme Reaction

To duplicate wells add 15 µL of P450 enzyme system (equivalent molar ratios of 2B4 P450, Cytochrome P450 Oxidoreductase, and Cytochrome b5 in a pre-sonicated 0.66 mg/mL DLPC solution), followed by 75 µL of the supplied Assay Buffer and 5 µL of P450 substrate. Seal the plate and incubate for 5 minutes at 37°C prior to addition of 5 µL of the reconstituted supplied NADPH activator. Seal the plate again and incubate for 15 minutes at 37°C. Add 5 µL of the supplied Stop Solution followed by the addition of 25 µL of the FDR to each well. Reseal the plate and incubate at 37°C for 30 minutes. For calibration purposes to formaldehyde, the 15 µL of P450 enzyme solution is replaced with standards made from the supplied Formaldehyde stock.

For calibration purposes to formaldehyde, the P450 enzyme solution is replaced with standards made from the supplied Formaldehyde stock.

REACTION OVERVIEW

P450 Reaction

1. **Carry out demethylating enzyme reaction.**
2. **Stop the reaction (optimal), add FDR.**

Formaldehyde Detection

3. **Incubate at 37°C for 30 minutes, read signal.**
4. **Calibrate to Formaldehyde generated.**

REAGENT PREPARATION

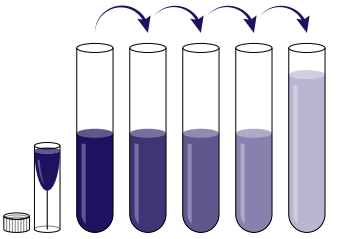
Allow the kit reagents to come to room temperature for 30 minutes. Ensure that all samples have reached optimal temperature for the P450 reaction and have been diluted as appropriate prior to running them in the kit.

NADPH Preparation

Allow the ziploc pouch to warm **completely** to room temperature prior to opening and remove a vial of NADPH. Add 600 µL of the Assay Buffer to the vial and vortex thoroughly. Store any unused reconstituted NADPH at ≤ -20°C for no more than 2 weeks.

Formaldehyde Standard Preparation

Label glass test tubes as #1 through #6. Pipet 400 µL of Assay Buffer into tube #1 and 250 µL into tubes #2-#6. Add 100 µL of the Formaldehyde stock solution to tube #1 and vortex completely. Add 250 µL of tube #1 to tube #2 and vortex completely. Repeat the serial dilutions for tubes #3 through #6. The concentration of formaldehyde in tubes 1 through 6 will be 400, 200, 100, 50, 25, and 12.5 µM.



Use all Standards within 2 hour of preparation.

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Buffer Volume (µL)	**400**	250	250	250	250	250
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5
Volume of Addition (µL)	**100**	250	250	250	250	250
Final Conc (µM)	400	200	100	50	25	12.5

ASSAY PROTOCOL

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine P450 activity.

P450 reaction volume should be no more than 100 µL in each well including all cofactors, inhibitors and activators so that 25 µL of FDR can be added to each well for detection.

1. Use the plate layout sheet on the back page of the insert to aid in proper sample and standard identification. Set plate parameters for a 96-well Corning Costar 3694 plate.
   See: www.ArborAssays.com/resources/#general-info for plate dimension data.

P450 Reaction

2. Pipet 95 µL of Assay Buffer as a Zero standard, standards or samples including all cofactors, substrates and/or inhibitors into the duplicate wells in the black plate. Seal with the plate sealer and incubate for 15 minutes at 37°C.

3. Add 5 µL of the reconstituted NADPH to each well, seal the plate and incubate at 37°C for 15-60 minutes (incubation time varies and is based upon the system and microsomes used - see pages 7 and 13).

4. Add 5 µL of Stop Solution to each well.

Formaldehyde Detection

5. Add 25 µL of the DetectX® Formaldehyde Detection Reagent to each well using a repeater pipet.

6. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Reseal with the plate sealer.

7. Incubate at 37°C for 30 minutes. Room temperature incubation will yield approximately 75% of the fluorescent signal generated with 37°C incubation.

8. Read the fluorescent signal from each well in a plate reader capable of reading the fluorescent signal at 510 nm with excitation at 450 nm. Please contact your plate reader manufacturer for suitable filter sets. **This assay requires a plate reader with efficient fluorescence optics. Please refer to page 6 for details on increasing sensitivity.**

CALCULATION OF RESULTS

Average the duplicate FLU readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean FLUs for the zero standard. The sample activity obtained should be multiplied by the dilution factor to obtain neat sample values.

Or use the online tool from MyAssays to calculate the data:
www.myassays.com/arbor-assays-p450-demethylating-fluorescent-activity-kit.assay

TYPICAL DATA

Cyp 2B4 P450 Demethylation of Benzphetamine

Low Sensitivity (Gain Setting at Low)

pmoles/100µL of P450 System	Mean FLU	Net FLU
20	32,755	31,576
16	30,879	29,700
12	26,296	25,117
8	20,523	19,344
4	10,274	9,095
3.2	7,577	6,399
2.4	4,751	3,573
1.6	2,184	1,006
0	1,179	0

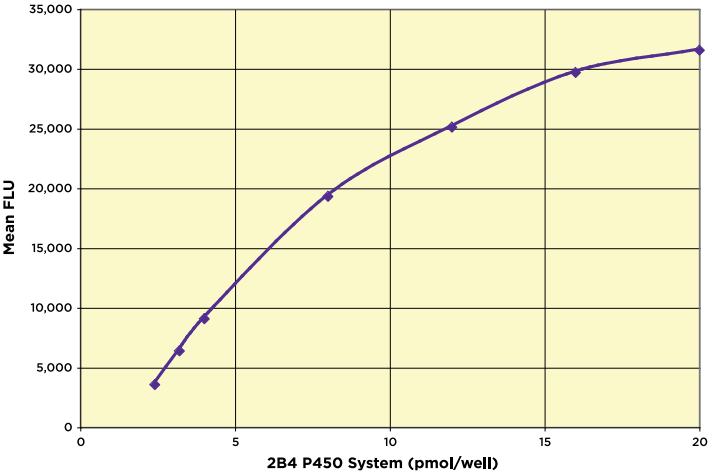
High Sensitivity (Gain Setting at High)

pmoles/100µL of P450 System	Mean FLU	Net FLU
4	41,400	35,982
3.2	28,876	23,459
2.4	19,429	14,012
1.6	9,283	3,865
0.8	6,077	659
0	5,418	0

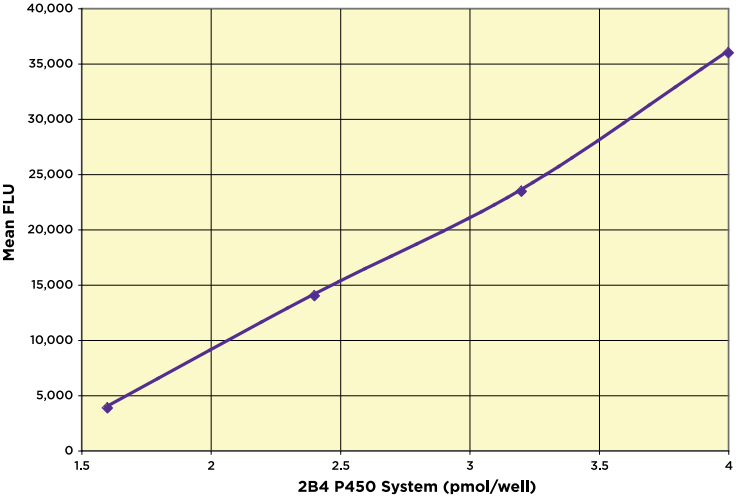
Typical Standard Curve

Typical 2B4 P450 Standard Curve

Low Sensitivity - (Plate Reader Gain Set on Low)



High Sensitivity - (Plate Reader Gain Set on High)



Always run your own standard curve for calculation of results. Do not use this data.

INTERFERENCE STUDIES

The following additives were added to the 2B4 P450 enzyme reaction with its substrate to test for interference with the signal generation.

Solvent studies described here were carried out with a 62.5 mM potassium phosphate buffer at pH 7.4 containing 0.005% gentamicin. Preservative studies were carried out in the same buffer with various added preservative concentrations.

Phosphate buffers with molarities up to 0.5M with the 2B4 P450 system are compatible with the assay.

Organic Solvents

Additions of 1% and 5% of the organic solvents ethanol, methanol, N,N-dimethylformamide and dimethylsulfoxide were tested in the 2B4 P450 assay. DMF and DMSO at 5% and ethanol at 1% showed small negative effects on the signal (< 9% decrease). Methanol at 1% gave a 15% drop in signal and at 5% a 20% decrease in P450 generated signal.

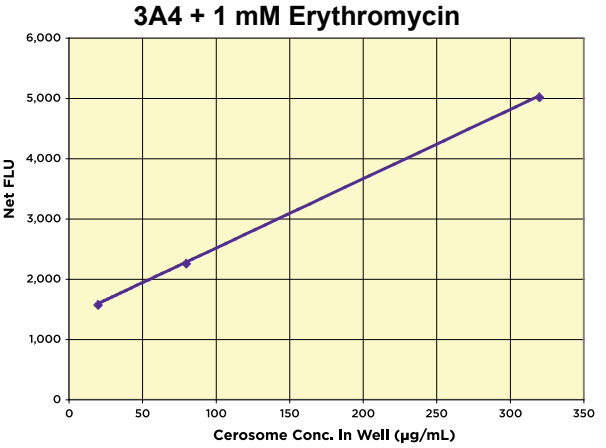
Preservatives

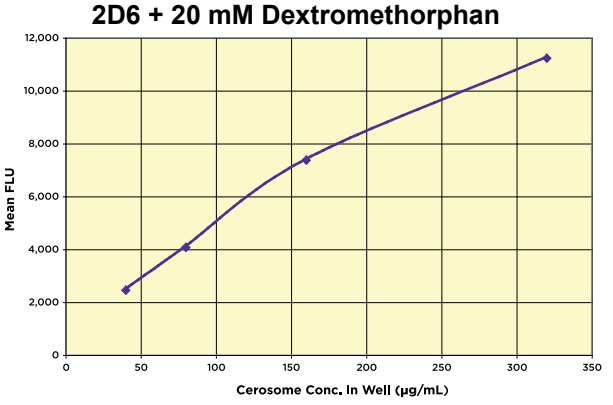
Sodium azide at 0.09% or gentamicin at 0.005% in the assay buffer increased the P450 generated signal by 20%. Kathon at 0.09% inhibited the signal by 83.2%.

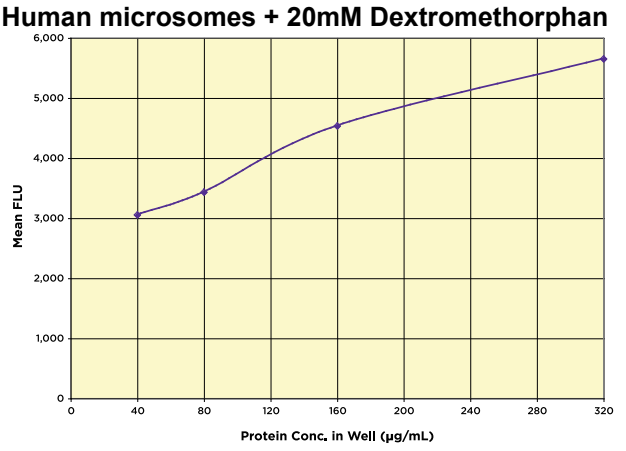
CEROSOME™/MICROSOME EXPERIMENTS

We tested the assay with a number of microsomal preparations using both rat and human liver native microsomes and with P450 co-expressed in Saccharomyces cerevisiae (CEROSOMES™, kind gift of PREMAS Biotech). In each case the microsomal preparation was incubated with substrate (1 mM erythromycin for 3A4, 20 mM dextromethorphan for 2D6 and 20 mM benzphetamine for 2B4) and NADPH at 37°C for 60 minutes and stopped using the provided Stop Solution. Production of formaldehyde from the individual P450 systems was then detected by addition of FDR and incubation at 37°C for 30 minutes. Fluorescence intensity was then measured.

Ceresome/Microsome Data



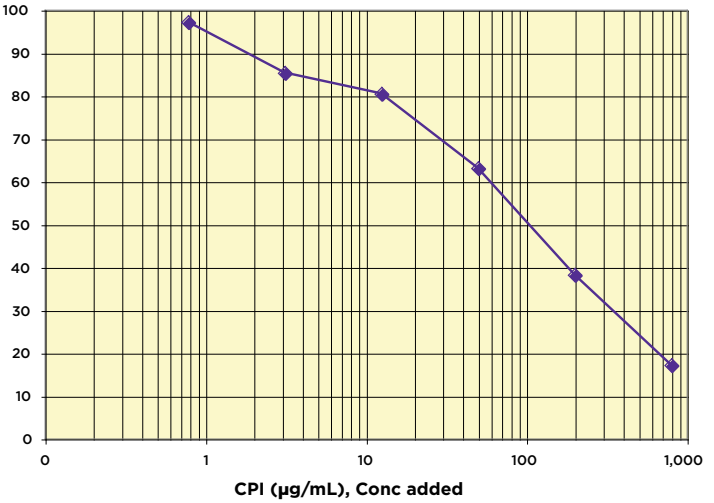




P450 INHIBITION EXPERIMENTS

Inhibition of 2B4 P450 Activity

The 2B4 P450 inhibitor, 1-chlorophenyl imidazole (CPI), was added to P450 enzyme system with its substrate at concentrations ranging from 0 to >200 µM in the microtiter plate well. The following graph shows the effect on 2B4 P450 signal.



LIMITED WARRANTY

Arbor Assays warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

CONTACT INFORMATION

For details concerning this kit or to order any of our products please contact us:

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OFFICIAL SUPPLIER TO ISWE

Arbor Assays and the International Society of Wildlife Endocrinology (ISWE) signed an exclusive agreement for Arbor Assays to supply ISWE members with EIA kits for wildlife conservation research.

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