Datasheet for ABIN577653

P450 Demethylation Activity Kit

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Overview

Quantity:	2 x 96 tests
Target:	Array
Minimum Detection Limit:	< 100 µU of GST Activity
Application:	Biochemical Assay (BCA)

Product Details

Purpose:	The DetectX® P450 Activity kit is designed to quantitatively measure the enzymatic activity of formaldehyde-producing enzymes such as Cytochrome P450s.
Brand:	DetectX®
Detection Method:	Fluorometric
Specificity:	Sample Types validated: Demethylating P450 systems: liver microsomes or cerosomes such as Cyp P450 3A4, 2B4 and 2D6
Characteristics:	The P450 Fluorescent Activity kit allows activity measurement of any demethylating P450 system WITHOUT any additions to the the P450:Substrate reaction. This assay measures the formaldehyde generated by demethylation and the signal is read AFTER the P450 reaction has been terminated. Convenient plate assay with 30 minute fluorescent substrate incubation and detection readout at 510 nm. Tested in 3A4, 2D6 and 2B4 P450 systems with erythromycin, dextromethorphan or benzphetamine. The cytochrome P450s (P450s) are a superfamily of heme containing enzymes that display tremendous diversity with regard to substrate specificity and catalytic activity. P450s use a plethora of both exogenous and endogenous compounds as substrates in their reactions. Usually they form part of multicomponent electron transfer reactions. 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 contains both FAD and FMN and can transfer both electrons needed for the catalytic cycle. In some P450 reactions, the second electron of the reaction cycle also can be delivered by cytochrome b5. The P450 enzymes and cofactors of the mammalian drug-metabolizing system are embedded in the membrane of the endoplasmic reticulum. The P450s play a crucial role in the development of new drug entities as drug interactions commonly inhibit cytochrome P450 activities.
Components:	Black Half Area 96 Well Plate Two plates Assay Buffer 60 mL A 100 mM potassium phosphate buffer at pH 7.4 containing 0.005% gentamicin. NADPH lyophilized 2 vials Reduced ß-nicotinamide adenine dinucleotide 2'-phosphate (NADPH) freeze dried with stabilizers and stored in desiccators. Stop Solution 1 mL A 1M solution of Acetic Acid in water. CAUTION: Acid solution. Formaldehyde Standard 0.5 mL 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 DetectX® Formaldehyde Reagent 5 mL Special formulation of reagents to detect formaldehyde in solution. Contains ≤ 0.09% sodium azide as a preservative. Plate Sealers 2 each
Material not included:	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 excita- tion at 450 nm. Set plate parameters for a 96-well Corning Costar 3694 plate. See: http://www. ArborAssays.com/resources/lit.asp 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 increas- ing 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.

Product Details

	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.

Target Details

Target:	Array
Background:	The cytochromes P450 (P450s) are a superfamily of heme containing enzymes that display tremendous diversity with regard to substrate specificity and cat- alytic activity1,2. P450s use a plethora of both exogenous and endogenous compounds as substrates in enzymatic reactions. Usually they form part of multicomponent electron transfer re- actions (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 (re- ductase) contains both FAD and FMN and can transfer both electrons needed for the catalytic cycle3. In some P450 reac- tions, the second electron of the reaction cycle also can be de- livered by cytochrome b54. The P450 enzymes and cofactors of the mammalian drug-metabolizing system are embedded in the membrane of the endoplasmic reticulum5. 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 pu- rification6. Most in vitro studies for the reconstitution of P450 activities use dilaurylphosphati- dylcholine (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 signifi- cantly7

Application Details

Application Notes:	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 detec- tion system

	and the ability to quantitate the formaldehyde produced by the Cyp 2B4 P450 enzy- matic reaction. Typical Cyp 2B4 Enzyme Reaction To duplicate wells add 15 µL of P450 enzyme system (equivalent molar ratios of 2B4 P450, Cy- tochrome P450 Oxidoreductase, and Cytochrome b5 in a pre-sonicated 0.66 mg/mL DLPC solu- tion), 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 25µL 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 solu- tion 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.
Protocol:	The kit is unique in that the fluo- rescent 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.
Reagent Preparation:	Allow the kit reagents to come to room temperature for 30 minutes. We recommend that all stan- dards and samples be run in duplicate to allow the end user to accurately determine P450 activity. 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 Remove a vial of NADPH from the desiccator and 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 six 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 com- pletely. 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 µ M. 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 Procedure:	1. 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. 2. Use the plate layout sheet on the back page of the insert to aid in proper sample and standard identification. P450 Reaction 3. 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

Application Details

	and incubate for 15 minutes at 37 °C. 4. 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). 5. Add 5µL of Stop Solution to each well. Formaldehyde Detection 6. Add 25 µL of the DetectX® Formaldehyde Detection Reagent to each well using a repeater pipet. 7. Gently tap the sides of the plate to ensure adequate mixing of the reagents. 8. Incubate at 37 °C for 30 minutes. Room temperature incubation will yield approximately 75 % of the fluorescent signal generated with 37 °C incubation. 9. Set plate parameters for a 96-well Corning Costar 3694 plate. See: http://www.ArborAssays.com/resources/lit.asp for plate dimension data. 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 fac- tor to obtain neat sample values.
Restrictions:	For Research Use only

Handling

Precaution of Use:	As with all such products, this kit should only be used by qualified personnel who have had labo- ratory safety instruction. The complete insert should be read and understood before attempting to use the product. 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.
Storage:	4 °C,RT
Storage Comment:	All components of this kit should be stored at 4°C until the expiration date of the kit.

Publications

Product cited in:	Talbot, Caperna, Garrett: "Growth and Development Symposium: Development, characterization, and use of a porcine epiblast-derived liver stem cell line: ARS-PICM-19." in: **Journal of animal science**, Vol. 91, Issue 1, pp. 66-77, (2013) (PubMed).

Images

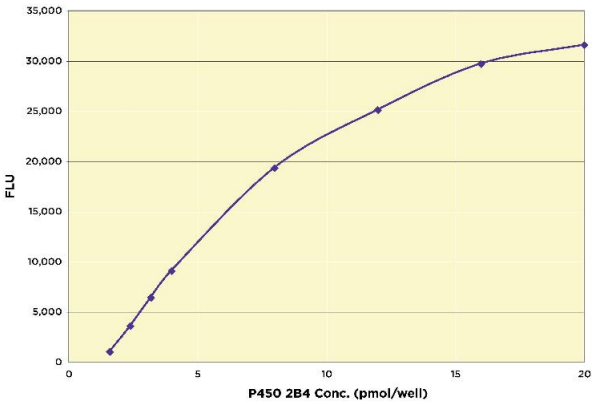

Image 1.

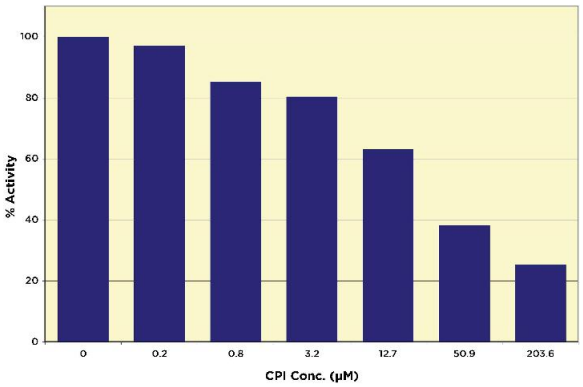

Image 2.