P450 Demethylation Fluorescent Activity Kit

Catalog Number EIAP450DMT (192 tests)

Rev 1.0

CAUTION! This kit contains materials with small quantities of sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state, and local regulations for disposal.

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *ELISA Technical Guide* (Pub. no. MAN0006706). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Product description

The P450 Demethylation Fluorescent Activity Kit is a fluorescent activity assay designed to measure cytochrome P450 activity in a variety of samples. The fluorescent reaction is initiated with the Formaldehyde Reagent which produces a fluorescent signal (450 nm excitation, 510 nm emission) when added to formaldehyde containing samples.

Cytochrome P450 acts on a number of exogenous and endogenous compounds as substrates in enzymatic reactions. Several cytochrome P450 systems are involved in demethylation, which produce formaldehyde as a byproduct of the reaction. This assay measures the activity of cytochrome P450 in demethylating systems like 3A4, 2B4, and 2D6 P450 systems in liver microsomes or recombinant microsomal preparations. The assay was validated with human cytochrome P450, but can be used with samples from other species.

Contents and storage

Kit and components are shipped at –20°C. Upon receipt, store the kit at –20°C. Once open, store the kit at 4°C and use within 2 weeks.

Components	Quantity
Formaldehyde Standard; 2,000 µM formaldehyde solution in deionized water, **keep tightly sealed**	500 µL
Assay Buffer; 100 mM potassium phosphate buffer at pH 7.4 containing 0.005% gentamicin	60 mL
Black 96-well Half Area Plate	2 plates
NADPH lyophilized; reduced ß-nicotinamide adenine dinucleotide 2'-phosphate (NADPH) with stabilizers	2 vials
Formaldehyde Reagent; contains 0.09% sodium azide as a preservative	5 mL
Stop Solution; 1M solution of acetic acid in water, **CAUTION**	1 mL
Plate Sealer	2

Materials required but not supplied

- Distilled or deionized water
- Fluorescence microtiter plate reader with software capable of measurement at or near 510 nm, with excitation at 450 nm
- 37°C incubator
- Calibrated adjustable precision pipettes and glass or plastic tubes for diluting solution
- Microsome, cerosome, baculosome, or supersome P450 systems; OR recombinant P450, NADPH/P450 oxidoreductase and cytochrome b5 system; dilaurylphosphatidylcholine (DLPC)

Procedural guidelines

- Review the **Procedural guidelines** and **Plate washing directions** in the *ELISA Technical Guide* available at **www.lifetechnologies.com/manuals** for details prior to starting the procedure.
- Reagents are lot-specific. Do not mix or interchange different reagent lots from various kit lots.
- Formaldehyde is a toxic, volatile, reactive chemical. Use in a well-ventilated laboratory. Dispose of all excess standards and samples in a 10% aqueous solution of sodium bisulfite, or according to the appropriate institutional guidelines.

Sample preparation guidelines

Important: Formaldehyde is a toxic, volatile, reactive chemical. Use in a well-ventilated laboratory. Dispose of all excess standards and samples in a 10% aqueous solution of sodium bisulfite, or according to the appropriate institutional guidelines.

Dilute samples

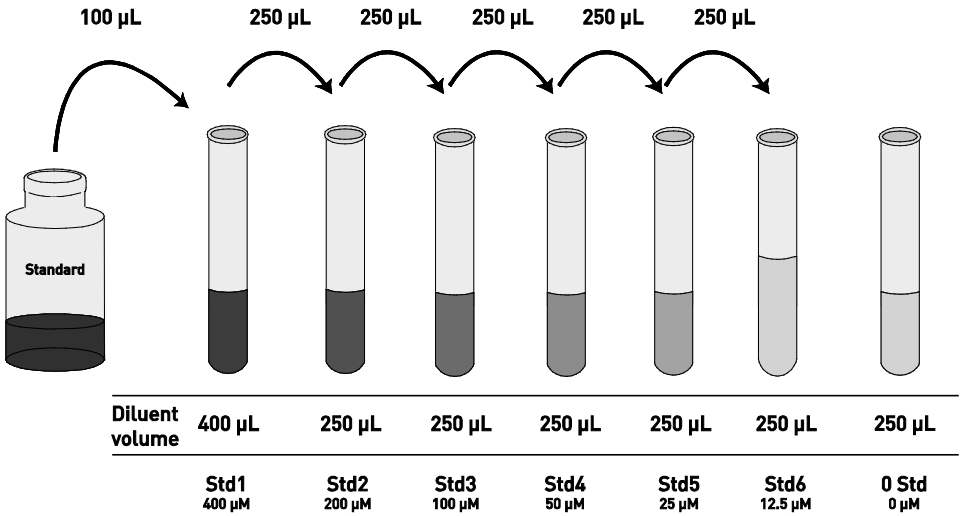
Sample concentrations should be within the range of the standard curve. Because conditions may vary, each investigator should determine the optimal dilution for each application.

Perform dilutions with the Assay Buffer supplied in the kit or a 0.1 M phsopahte buffer, pH 7.4.

Dilute standards

Note: Use glass or plastic tubes for diluting standards.

1. Add 100 µL Formaldehyde Standard to one tube containing 400 µL Assay Buffer and label as 400 µM formaldehyde.
2. Add 250 Assay Buffer to each of 6 tubes labeled as follows: 200, 100, 50, 25, 12.5, and 0 µM formaldehyde.
3. Make serial dilutions of the standard as described below in the dilution diagram. Mix thoroughly between steps.
4. **Use the standards within 2 hours of preparation**.



Reconstitute NADPH

1. Allow the NADPH to reach room temperature in the sealed bag before opening.
2. Add 600 µL of Assay Buffer to the vial of NADPH and vortex thoroughly.
3. Store any unused reconstituted NADPH at –20°C or lower for no **longer than 2 weeks**.

Assay procedure

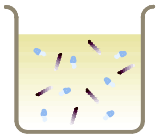
Allow all reagents to reach room temperature before use. Mix all liquid reagents prior to use. **Total assay time is 30 minutes.**

IMPORTANT! Perform a standard curve with each assay.

Guidelines for setting up the P450 reaction

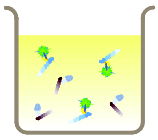
The assay detects the activity of the 2B4, 2D6, and 3A4 P450 systems. The conditions provided in this procedure are for the Cyp 2B4 P450 system, which was used in validating the assay.

- P450 reaction volume should be no more than 100 µL in each well including all cofactors, inhibitors, and activators.
- For a typical Cyp 2B4 enzyme reaction, add 15 µL of the 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) to 75 µL of Assay Buffer, and 5 µL of P450 substrate (benzphetamine).



Add P450 reaction

a. Set up the P450 reaction.
b. Add 95 µL of standards or diluted samples (see page 2) to the appropriate wells.
c. Cover the plate with a plate sealer and incubate for 15 minutes at 37°C. [1]
d. Add 5 µL of reconstituted NADPH to each well, reseal the plate and incubate at 37°C for 15–60 minutes. [1]
e. Add 5 µL of Stop Solution to each well.



Add fluorescent detection reagent

a. Add 25 µL of Formaldehyde Reagent to each well and reseal the plate.
b. Tap the side of the plate to mix.
c. Incubate for 30 minutes at 37°C.

[1] The incubation time varies and is based upon the system and microsomes used. Because conditions may vary, each investigator should determine the optimal time for each application.

Read the plate and generate the standard curve

1. Read the fluorescent emission at 510 nm, with excitation at 450 nm.
2. Use curve-fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit. Optimally, the background fluorescence may be subtracted from all data points, including standards, unknowns and controls, prior to plotting.
3. Read the activity of unknown samples and controls from the standard curve. Multiply value(s) obtained for sample(s) by the appropriate factor to correct for the sample dilution.

   Note: Dilute samples producing signals greater than that of the highest standard in appropriate diluent and reanalyze. Multiply the activity by the appropriate dilution factor.

Performance characteristics

Standard curve (example)

The following data were obtained for the various standards.

High sensitivity (gain setting at high)

Standard Formadehyde (pmoles/100 µL of P450 System)	Mean FLU
4	41,400
3.2	28,876
2.4	19,429
1.6	9,283
0.8	6,077
0	5,418

Low sensitivity (gain setting at low)

Standard Formadehyde (pmoles/100 µL of P450 System)	Mean FLU
20	32,755
16	30,879
12	26,296
8	20,523
4	10,274
3.2	7,577
2.4	4,751
1.6	2,184
0	1,179

Performance characteristics, continued

Interferents

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

- Phosphate buffers with molarities up to 0.5M with the 2B4 P450 system are compatible with the assay.
- 5% DMF and DMSO, and 1% ethanol showed small negative effects on the signal (<9% decrease). 1% methanol reduced signal by 15%, while 5% methanol reduced signal by 20%.
- 0.09% sodium azide or 0.005% gentamicin in the assay buffer increased the signal by 20%, while 0.09% Kathon™ inhibited the signal by 83.2%.

Limited product warranty

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