CYTOCHROME P450 2B FLUORESCENT DETECTION KIT

ProductInformation

Technical Bulletin No. **CYTO-2BTB**

TECHNICAL BULLETIN

Product Description

The function of the cytochrome P450 enzymes is to metabolize xenobiotic compounds with which an organism comes into contact. These enzymes are found mainly in the mammalian liver (although they are also found in many other organisms as well)[1]. This metabolism is achieved through a mixed monooxygenase system with the general EC number of 1.14.14.1[2]

The cytochrome P450 enzymes are divided into four major groups as shown below in Table 1. Each group of enzymes has different inducing agents. The specific reaction measured for the 2B4 activity is pentoxyresorufin O-dealkylation.

Table 1: Forms of Cytochrome P450

Family	Subfamily	Substrate	Inducer	Examples of inducers
P450 1	Cyp1A1	Ethoxyresorufin	Polycyclic hydrocarbons, dioxins	Benzo(a)pyrene, 3-methylcholanthrene, β-naphthoflavone
	Cyp1A2	Methoxyresorufin	Polycyclic hydrocarbons, dioxins, isosafrole	As above for 1A1
P450 2	**Cyp2B1/2/4**	**Pentoxyresorufin**	**Barbiturates, chlorinated hydrocarbons and aromatics**	**Phenobarbital**
	Cyp2E1	Aniline • HCl	Ethanol, isoniazid, acetone, imidazole	
P450 3	Cyp3A1		Pregnenolone-16α-carbonitrile	
P450 4	Cyp4A1		Clofibrate, phthalates	

The cytochrome P450 enzymes have molecular weights of 45-60 kDa (SDS-PAGE) depending on the inducer and the animal source[3]. The molecular weight of cytochrome 2B4 has been shown to be approximately 49 kDa[3,4].

Crude cytochrome P450 enzyme preparations may be used as standards for determining the presence of the different forms of cytochrome P450 in the liver. The profile of the various forms is indicative of the exposure of the organism to inducing agents from the environment. 2B enzymes in the mammalian liver are induced by such substances as barbiturates.

Reagents Provided

This kit enables the extraction of unknown samples and the assay of Cytochrome P450 2B activity. Reagents are sufficient for the extraction of a total of 7.5 g of tissue and up to 50 assays.

Components	Amount
10X Tissue Extraction Buffer, Product No.T9407	25 ml
200X Inhibitor Solution, Product No. I0515	1.25 ml
100X $CaCl_2$ Solution, Product No. C3970	2 ml
Cytochrome P450 Assay Buffer, Product No. C9306	100 ml
1 mM Pentoxyresorufin Substrate Solution, Product No. P9438	1 ml

Components	Amount
1 mM Resorufin Standard Solution, Product No. R9511	0.1 ml
NADPH, Product No. 201-205	5 x 5 mg
Cytochrome P450 2B4, Product No. C7552 Positive control induced in rabbit liver by phenobarbital **Activity:** 150-450 units per mg protein **Unit definition:** One unit will release 1.0 pmole of resorufin from pentoxyresorufin (P0928) per min at pH 7.6 at 37°C.	2 x 0.5 mg protein

Principle of the Assay

This assay is based on the fact that cytochrome P450 can hydrolyze an alkyloxyresorufin substrate that is not fluorescent to the fluorescent compound resorufin[5], which can be detected using a spectrofluorometer. This reaction requires the presence of cytochrome P450, cytochrome P450 reductase and cytochrome b_5 which are all found in the microsomal fraction as well as the addition of the extrinsic cofactor NADPH. The fluorescent assay is a modified method essentially described by Boulenc, et al.[6], and is based on previous work by Burke and Mayer[7]. It is important to note that the amount (µg protein) of enzyme in the reaction is critical. Too high a concentration of the enzyme will seriously underestimate the activity present[7].

Enzyme Unit Definition: One unit of enzyme activity will release 1.0 pmole of resorufin from pentoxy-resorufin (P0928) per min at pH 7.6 at 37°C.

Precautions and Disclaimer

For laboratory use only. Not for drug, household or other uses.

Sample Preparation Procedure

In order to test a specific organism for the presence of cytochrome P450 2B, it is necessary to prepare a microsomal fraction from the liver of the organism.

Reagent and Working Buffer Preparations

1) 10X Tissue Extraction Buffer, T9407

500 mM HEPES/NaOH pH 7.9 + 1 M KCl + 10 mM EDTA

Dissolve the vial in deionized water to a final volume of 25 ml.

Alternately, weigh out 1 g and dissolve in a total volume of 4.8 ml deionized water.

Note: The pH of the concentrated solution will be approximately pH 7.9, but upon dilution to the working concentration it will be pH 7.5.

2) 200X Inhibitor Solution, I0515

200 mM AEBSF + 4.6 mM Butylated Hydroxytoluene in DMSO

3) 100X $CaCl_2$ Solution, C3970

800 mM $CaCl_2$

Dissolve the contents of the vial in 2 ml of deionized water.

4) WB1 - Working Buffer 1 (Prepared from **T9407** and **I0515**)

50 mM HEPES/NaOH pH 7.5 + 100 mM KCl + 1 mM EDTA + 1 mM AEBSF + 23 µM Butylated Hydroxytoluene

Prepare as follows: In a beaker add 10 ml of 10X Tissue Extraction Buffer (T9407) and 80 ml of deionized water. Stir and add 0.5 ml of Inhibitor Solution (I0515). Bring to a final volume of 100 ml with deionized water. This solution is stable at 4°C for at least 24 hours.

5) WB2 - Working Buffer 2 (Prepared from **T9407**, **I0515** and **C3970**)

50 mM HEPES/NaOH pH 7.5 + 100 mM KCl + 1 mM EDTA + 1 mM AEBSF + 23 mM Butylated Hydroxytoluene + 8 mM $CaCl_2$.

Prepare as follows: In a beaker add 1 ml of 800 mM $CaCl_2$ Solution (C3970) and 10 ml of 10X Tissue Extraction Buffer (T9407). Add 80 ml of deionized water and stir well. Add 0.5 ml of Inhibitor Solution (I0515). Bring to a final volume of 100 ml with deionized water and mix well. This solution is stable at 4°C for at least 24 hours.

Microsomal Fraction Preparation Procedures

Method 1 - Procedure with an Ultracentrifuge

Microsomes may be prepared according to the procedure listed below. The following **equipment** is required but not provided.

a) Sorvall RC-5C centrifuge with SS-34 head or equivalent.

b) Ultracentrifuge with 12 ml tubes.

c) Ultra-Turrax T25 homogenizer with S 25N-8G head.

d) 80 mesh screen for tissue filtration

STEP 1.1) With gentle agitation wash 1-2 grams of liver tissue 5 times with 3 volumes (3-6 ml) of **WB1**. Each time remove the red colored liquid by filtration of the tissue on a fine screen or by decanting.

1.2) Homogenize the tissue in 4 volumes (4-8 ml) of **WB1** using an UltraTurrax T25 homogenizer with S 25N-8G head.

1.3) Centrifuge the homogenate in a Sorvall RC-5C centrifuge with SS-34 head at 4°C for 30 minutes at 10,000 rpm (RCF ≅ 7,700x*g*) using 35 ml polypropylene tubes.

1.4) Carefully remove and save the supernatant fluid and then rehomogenize the pellet with 2 volumes (2-4 ml) of **WB1** (The pellet can be rehomogenized directly in the tube.)

1.5) Centrifuge as in **step 1.3**.

1.6) Pool both supernatants (Volume ≅ 6-12 ml).

1.7) Bring the supernatant pool to 8 mM in $CaCl_2$ by addition of 10 µl of the 800 mM $CaCl_2$ Solution (C3970) per ml of supernatant.

1.8) Mix for 15-30 minutes and then centrifuge at 4°C for 30 minutes at RCF ≅ 100,000x*g* in an ultracentrifuge.

1.9) Discard the supernatant fluid and wash the pellet once with 4 volumes (4-8 ml) of **WB2** by homogenizing as before in **step 1.2** and centrifuging as in **step 1.8**.

1.10) Discard the supernatant and resuspend the pellet in 4 ml of **WB2** by homogenization.

1.11) Assay the microsomal suspension for cytochrome P450 2B activity as detailed below.

Method 2 - Procedure without an Ultracentrifuge

In the event that the ultracentrifuge used in Method 1 is not available it is possible to prepare the microsomal suspension by the following method. Preparation of the enzyme with this method ***may*** underestimate the amount of the cytochrome P450 by up to 50% due to poor sedimentation of the microsomal fraction at the low *g* forces used here.

The following **equipment** is required but not provided.

a) Eppendorf centrifuge Model 5415 C or equivalent (Maximum RCF ≅ 16,000x*g*) and 2 ml Eppendorf tubes.

b) Teflon and glass tissue grinder.

STEP 2.1) Wash with gentle agitation 1-2 grams of liver tissue 5 times with 3 volumes (3-6 ml) of **WB1**. Each time remove the red colored wash by filtration of the tissue on a fine screen or by decantation.

2.2) Homogenize the tissue in 4 volumes (4-8 ml) of **WB1** using a teflon and glass tissue grinder.

2.3) Centrifuge the homogenate in an Eppendorf centrifuge at 4°C for 30 minutes at 9,500 rpm (RCF ≅ 7,250x*g*) using 2 ml Eppendorf tubes.

2.4) Carefully remove and save the supernatant fluid and then rehomogenize the pellet with 2 volumes (2-4 ml) of **WB1**.

2.5) Centrifuge as in **step 2.3**.

2.6) Pool both supernatants (Volume ≅ 6-12 ml).

2.7) Bring the supernatant pool to 8 mM in $CaCl_2$ by addition of 10 µl of the 800 mM $CaCl_2$ Solution (C3970) per ml of supernatant.

2.8) Mix for 15-30 minutes and then centrifuge at 4°C for 60 minutes at 14,000 rpm (RCF ≅ 16,000x*g*) using 2 ml Eppendorf tubes.

2.9) Discard the supernatant, suspend the pellet in 1 ml of **WB2** per tube and homogenize as before in **step 2.2**.

2.10) Centrifuge as in **step 2.8**.

2.11) Discard the supernatant, resuspend the pellet in 1 ml of **WB2** per tube and then pool all tubes.

2.12) Assay the suspension for cytochrome P450 2B activity as detailed below.

Fluorescent Assay Procedure

Equipment needed
Spectrofluorometer with a stirred thermostatted cell

Reagents
1) Cytochrome P450 Assay Buffer, C9306

100 mM Na/K-Phosphate buffer pH 7.6 + 100 mM KCl + 2.5 mM $MgCl_2$

Dissolve the whole bottle to a final volume of 100 ml deionized water, or weigh out 0.5 g solid and dissolve in 21.3 ml deionized water. This solution is stable for at least 30 days at room temperature.

2) NADPH, 201-205

Dissolve the 5 mg vial in 1.25 ml of deionized water. This gives a 5 mM solution. Prepare ***fresh*** each day.

3) Pentoxyresorufin Substrate Solution, P9438

Pentoxyresorufin (P0928) 1 mM; 0.284mg/ml in DMSO

4) Resorufin Standard Solution, R9511

Resorufin 1 mM; 0.235 mg/ml in DMSO

To prepare a standard curve, dilute the Standard Solution 400 times with deionized water to a concentration of 2.5 µM.

5) Positive control of cytochrome P450 2B4, C7552

Add 0.5 ml of deionized water to the vial. This will give a suspension of approximately 1 mg protein per ml. This solution can be aliquoted and frozen at -70°C to preserve the activity.

Procedure

1) Preincubate the Assay Buffer at 37°C.
2) Place 1.8 ml of Assay Buffer in a stirred fluorescence cuvette and place in a spectrofluorometer thermostatted at 37°C.
3) Set the fluorometer excitation wavelength to 530 nm and emission wavelength to 585 nm. Set the bandwidth to 5 nm.
4) Add to the cuvette 20 µl of the pentoxyresorufin substrate solution.
5) Add 30 to 90 µg of sample microsomal enzyme protein to the cuvette.

Note: Very low and very high levels of the enzyme may give abnormal results, with too high a concentration of the enzyme seriously underestimating the activity present so it is recommended to test the activity at several levels of protein concentration.[7]

6) Start the reaction by addition of 100 µl of NADPH solution.
7) Measure the Fluorescence Units (FLU) every minute for 10 minutes.
8) Calculate the ΔFLU/min from the linear portion of the curve. In most cases this will lie between 1 to 5 minutes; however, in some cases a lag in the activity up to several minutes may be observed.
9) Calibrate the fluorometer using the prepared resorufin curve. Using the prepared 2.5 µM solution, add 2.5, 5, 10, 25, 50 and 100 µl to 2 ml of the Assay Buffer. Read the fluorescence and draw a standard curve. This curve represents 6.25, 12.5, 25, 62.5, 125 and 250 pmole of resorufin.

Calculation

Determine the value of Fluorescent Units (FLU) per pmole of product from the resorufin standard curve.

$$\text{1 unit of activity (pmole/ml/min)} = \frac{\Delta\text{FLU/min x dilution factor of sample}}{\text{(FLU/pmole) x (reaction volume [ml])}}$$

References

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