3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

Cytochrome P450 1A Fluorescent Detection Kit

Product Code **CYTO-1A**
Technical Bulletin No. **CYTO-1ATB**

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 mono-oxygenase system with the general EC# 1.14.14.1.[2]

The cytochrome P450 enzymes are divided into four families (Table 1). Each family of enzymes has different inducing agents. The specific reaction measured for the 1A1 activity is ethoxyresorufin O-dealkylation and the reaction measured for the 1A2 activity is methoxyresorufin O-dealkylation.

Table 1
Cytochrome P450 Families

Family	Subfamily	Substrate	Inducer	Examples of inducers
P450 1	**Cyp1A1**	**Ethoxyresorufin**	**Polycyclic hydrocarbons, dioxins**	**Benzo(a)pyrene, 3-methylcholanthrene, β-naphthoflavone and 2,3,7,8-tetrachloro-dibenzo-p-dioxin**
	Cyp1A2	**Methoxyresorufin**	**Polycyclic hydrocarbons, dioxins, isosafrole**	**Benzo(a)pyrene, 3-methylcholanthrene, β-naphthoflavone and isosafrole**
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 masses in the range of 45 to 60 kDa (SDS-PAGE) depending on the inducer and the animal source.[3] The molecular mass of cytochrome 1A1 from rabbit liver has been shown to be approximately 57.5 kDa and for cytochrome 1A2, approximately 54 kDa.[3,4] Levels of enzyme activity of 150 pmol/min/mg protein have been observed in rabbit livers after induction with β-naphthoflavone when using ethoxyresorufin as the substrate.[5]

Crude cytochrome P450 enzymes may be used as standards for determining the presence of different families 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. 1A enzymes in the mammalian liver are induced by such substances as dioxins and polycyclic hydrocarbons.

Reagents Provided

This kit provides for the extraction of unknown samples and the assay of Cytochrome P450 1A activity. Reagents are sufficient for the extraction of a total of 7.5 g of tissue and up to 50 assays.

Reagent	Amount
10x Tissue Extraction Buffer, Product Code T 9407	25 ml
200x Inhibitor Solution, Product Code I 0515	1.25 ml
$CaCl_2$, dihydrate Product Code C 3881	370 mg
Cytochrome P450 Assay Buffer, Product Code C 9306	100 ml
1 mM Methoxyresorufin Substrate Solution, Product Code M 4050	1 ml
1 mM Ethoxyresorufin Substrate Solution, Product Code E 1023	1 ml
1 mM Resorufin Standard Solution, Product Code R 9511	0.1 ml
NADPH, Product Code 201-205	5 x 5 mg
Cytochrome P450 1A1/2, Product Code C 9556 Positive control induced in rabbit liver by β-Naphthoflavone Activity: 30-120 units per mg protein Unit Definition: One unit will release 1.0 pmole of resorufin from ethoxyresorufin (E 3763) or methoxy-resorufin (M 1544) per min at pH 7.6 at 37 °C.	2 x 1.0 mg protein

Equipment Required but Not Provided

- Spectrofluorometer with a stirred, thermostatted cell

  And
- Sorvall® RC-5C centrifuge with SS-34 head or equivalent
- Ultracentrifuge with 12 ml tubes
- Ultra-Turrax® T25 homogenizer with S 25N-8G head
- 80 mesh screen for tissue filtration

  Or
- Eppendorf® centrifuge Model 5415 C or equivalent (Maximum RCF of approximately 16,000 x *g*) and 2 ml Eppendorf tubes
- Teflon and glass tissue grinder

Precautions and Disclaimer

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

Preparation Instructions

Reagent and Working Buffer Preparations

1. 10x Tissue Extraction Buffer - 500 mM HEPES, pH 7.9, with 1 M KCl and 10 mM EDTA
   Dissolve the contents of the vial (Product Code T 9407), 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 of 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 (Product Code I 0515) – DMSO solution with 200 mM AEBSF and 4.6 mM butylated hydroxytoluene. Solution is supplied ready to use.
3. 100x $CaCl_2$ Solution (800 mM $CaCl_2$) - Dissolve the contents of the vial (Product Code C 3881) in 2 ml of deionized water.
4. Working Buffer 1 (WB1) - 50 mM HEPES, pH 7.5, with 100 mM KCl, 1 mM EDTA , 1 mM AEBSF, 23 µM butylated hydroxytoluene. Prepared from 10x Tissue Extraction Buffer and 200x Inhibitor Solution. In a beaker, add 10 ml of 10x Tissue Extraction Buffer and 80 ml of deionized water. Mix and add 0.5 ml of 200x Inhibitor Solution. Bring to a final volume of 100 ml with deionized water. This solution is stable at 4 °C for at least 24 hours.
5. Working Buffer 2 (WB2) - 50 mM HEPES, pH 7.5, with 100 mM KCl, 1 mM EDTA, 1 mM AEBSF, 23 µM butylated hydroxytoluene, and 8 mM $CaCl_2$. Prepared from 10x Tissue Extraction Buffer, 200x Inhibitor Solution, and 100x $CaCl_2$ Solution. In a beaker, add 1 ml of 100x $CaCl_2$ Solution and 10 ml of 10x Tissue Extraction Buffer. Add 80 ml of deionized water and stir well. Add 0.5 ml of 200x Inhibitor Solution. 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.
6. Cytochrome P450 Assay Buffer - 100 mM Na/K-phosphate buffer, pH 7.6, with 100 mM KCl and 2.5 mM $MgCl_2$ - Dissolve the whole bottle (Product Code C 9306) to a final volume of 100 ml with deionized water, or weigh out 0.5 g of solid and dissolve in 21.3 ml of deionized water. This solution is stable for at least 30 days at room temperature.
7. NADPH Solution - 5 mM - Dissolve the 5 mg vial (Product Code 201-205) in 1.25 ml of deionized water. Prepare **fresh** each day.

8. Ethoxyresorufin Substrate Solution (Product Code E 1023) – DMSO solution of 1 mM (0.241 mg/ml) ethoxyresorufin (Product Code E 3763). Solution is supplied ready to use.
9. Methoxyresorufin Substrate Solution (Product Code M 4050) - DMSO solution of 1 mM (0.228 mg/ml) methoxyresorufin (Product Code M 1544). Solution is supplied ready to use.
10. Resorufin Standard Solution (Product Code R 9511) - DMSO solution of 1 mM (0.235 mg/ml) Resorufin. Solution is supplied ready to use. Note: To prepare a standard curve, dilute the Resorufin Standard Solution 400-fold with deionized water to a concentration of 2.5 μM.
11. Cytochrome P450 1A1/2 Positive Control - Add 0.5 ml of deionized water to the vial (Product Code C 9556). This will give a suspension of approximately 2 mg protein per ml. This suspension can be aliquoted and frozen at –70 °C to preserve the activity.

Procedure

This assay is based on the fact that cytochrome P450 can hydrolyze a non-fluorescent alkyloxyresorufin substrate to the fluorescent compound resorufin,[6] 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. This fluorescent assay is a modification of published procedures.[7,8] It is important to note that the amount of enzyme (μg of protein) in the reaction is critical. Too high a concentration of the enzyme will seriously underestimate the activity present.[8]

Microsomal Fraction Preparation Procedures

In order to test a specific organism for the presence of cytochrome P450 1A enzymes, it is necessary to prepare a microsomal fraction from the liver of the organism.

Method 1 - Procedure with an Ultracentrifuge

1.1 With gentle agitation, wash 1 to 2 g of liver tissue five times with 3 volumes (3 to 6 ml) of Working Buffer 1 (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 four volumes (4 to 8 ml) of WB1 using an Ultra-Turrax T25 homogenizer with S 25N-8G head.
1.3 Centrifuge the homogenate at 7,700 x *g* at 4 °C for 30 minutes (Sorvall RC-5C centrifuge with SS-34 head at 10,000 rpm using 35 ml polypropylene tubes).
1.4 Carefully remove and save the supernatant. Then rehomogenize the pellet with two volumes (2 to 4 ml) of WB1. The pellet may be rehomogenized directly in the tube.
1.5 Centrifuge as in step 1.3. Carefully remove and save the supernatant.
1.6 Pool the supernatants from steps 1.4 and 1.5. The volume is approximately 6 to 12 ml.
1.7 Adjust the final $CaCl_2$ concentration of the combined supernatants to 8 mM by addition of 10 μl of the 100x mM $CaCl_2$ Solution per ml of supernatant.
1.8 Mix for 15 to 30 minutes and then centrifuge at 100,000 x *g* at 4 °C for 30 minutes in an ultracentrifuge.
1.9 Discard the supernatant. Resuspend the pellet in four volumes (4 to 8 ml) of Working Buffer 2 (WB2) by homogenizing as in step 1.2. Centrifuge 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 1A activity (Fluorescent Assay Procedure).

Method 2 - Procedure without an Ultracentrifuge

In the event an ultracentrifuge required for 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.

2.1 With gentle agitation, wash 1 to 2 g of liver tissue five times with 3 volumes (3 to 6 ml) of Working Buffer 1 (WB1). Each time remove the red colored liquid by filtration of the tissue on a fine screen or by decanting.
2.2 Homogenize the tissue in four volumes (4 to 8 ml) of WB1 using a teflon and glass tissue grinder.
2.3 Centrifuge the homogenate at 7,250 x *g* at 4 °C for 30 minutes. (Eppendorf centrifuge Model 5415 C at 9,500 rpm using 2 ml Eppendorf tubes)
2.4 Carefully remove and save the supernatant. Then rehomogenize the pellet with two volumes (2 to 4 ml) of WB1.

2.5 Centrifuge as in step 2.3. Carefully remove and save the supernatant.
2.6 Pool the supernatants from steps 2.4 and 2.5. The volume is approximately 6 to 12 ml.
2.7 Adjust the final $CaCl_2$ concentration of the combined supernatants to 8 mM by addition of 10 µl of the 100x mM $CaCl_2$ Solution per ml of supernatant.
2.8 Mix for 15 to 30 minutes and then centrifuge at 16,000 x *g* at 4 °C for 60 minutes using 2 ml Eppendorf tubes.
2.9 Discard the supernatant. Resuspend each pellet in 1 ml of Working Buffer 2 (WB2) per tube by homogenizing as in step 2.2. Centrifuge as in step 2.8.
2.10 Discard the supernatant, resuspend each pellet in 1 ml of WB2 per tube, and then pool all tubes.
2.11 Assay the suspension for cytochrome P450 1A activity (Fluorescent Assay Procedure).

Fluorescent Assay Procedure

1. Preincubate the Cytochrome P450 Assay Buffer at 37 °C.
2. Place 1.8 ml of the Cytochrome P450 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 the emission wavelength to 585 nm. Set the bandwidth to 5 nm.
4. Add to the cuvette, 20 µl of the Ethoxy or Methoxyresorufin Substrate Solution.
5. Add a sample volume equivalent to 30 to100 µg of microsomal protein to the cuvette.
   Note: Very low and very high levels of the enzyme in the reaction mixture may give abnormal results. Too high of a concentration of enzyme may seriously underestimate the activity present. It is recommended to assay the activity at several levels of protein concentration.[8]
6. Start the reaction by addition of 100 µl of NADPH solution.
7. Measure the Fluorescent Units (FLU) every minute for up to 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 diluted resorufin solution. Using the prepared 2.5 µM solution, add 2.5, 5, 10, 25, 50, and 100 µl to 2 ml of Cytochrome P450 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 Flourescent Units (FLU) per pmole of product from the resorufin standard curve.

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

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