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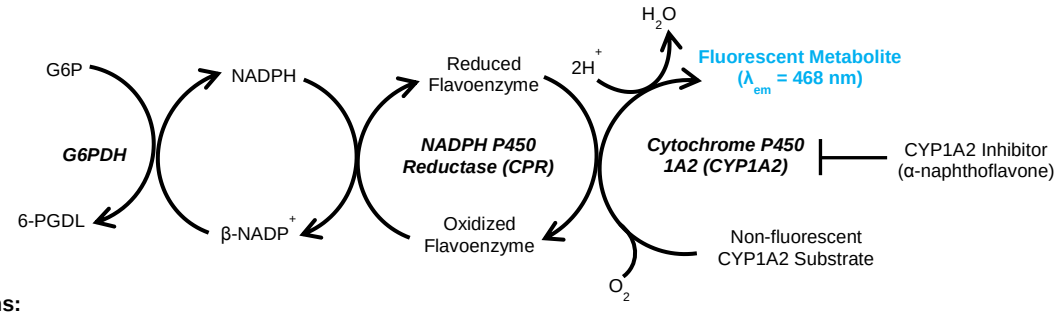
Cytochrome P450 1A2 (CYP1A2) Activity Assay Kit (Fluorometric)

(Catalog # K893-100; 100 Reactions; Store at -20°C) *rev 06/19*

I. Introduction:

Cytochrome P450 1A2 (CYP1A2, EC 1.14.14.1) is a member of the cytochrome P450 monooxidase (CYP) family of microsomal xenobiotic metabolism enzymes. CYPs are membrane-bound hemeproteins responsible for Phase I biotransformation reactions, in which lipophilic drugs and other xenobiotic compounds are converted to more hydrophilic products to facilitate excretion from the body. CYP1A2 is primarily expressed in liver, intestinal and olfactory mucosal tissue and catalyzes oxidation of polyaromatic and heterocyclic molecules such as aromatic amines. CYP1A2 is responsible for metabolism of approximately 10% of all small molecule drugs commonly used by humans. Polymorphisms in the human CYP1A2 gene have been implicated in clinical drug/drug interactions involving widely-used drugs, including caffeine, theophylline and the antipsychotic clozapine. Isoforms of the CYP1A subfamily are also involved in metabolic activation of environmental pro-carcinogens present in cigarette smoke and combustion exhaust fumes. BioVision's CYP1A2 Activity Assay Kit enables rapid measurement of native or recombinant CYP1A2 activity in biological samples such as liver microsomes. The assay utilizes a non-fluorescent CYP1A2 substrate that is converted into a highly fluorescent metabolite detected in the visible range (Ex/Em = 406/468 nm), ensuring a high signal-to-background ratio with little interference by autofluorescence. A selective CYP1A2 inhibitor is provided for determination of CYP1A2 activity in heterogeneous biological samples, where other CYP isozymes may contribute to substrate metabolism. The inhibitor displays greater than 20-fold selectivity for CYP1A2 over other CYPs, ensuring targeted inhibition. CYP1A2 specific activity is calculated by running parallel reactions in the presence and absence of the selective inhibitor and subtracting any residual activity detected with the inhibitor present. The kit contains a complete set of reagents sufficient for performing 50 sets of paired reactions (in the presence and absence of inhibitor).



II. Applications:

- Rapid assessment of native/recombinant CYP1A2 activity in fractions prepared from tissues and cells.
- Screening of drugs and novel ligands for interaction with native/recombinant CYP1A2.

III. Sample Type:

- Human liver microsomes and liver S9 fractions
- Lysates of tissues and cultured cells, primary hepatocytes
- Heterologously expressed recombinant CYP1A2 preparations

IV. Kit Contents:

Components	K893-100	Cap Code	Part Number
CYP1A2 Assay Buffer	100 ml	NM	K893-100-1
3-CHC Standard	1 vial	Yellow	K893-100-2
CYP1A2 Inhibitor (α-naphthoflavone)	1 vial	Blue	K893-100-3
NADPH Generating System (100X)	1 vial	Green	K893-100-4
β-NADP$^+$ Stock (100X)	1 vial	Amber	K893-100-5
CYP1A2 Substrate	1 vial	Red	K893-100-6
Recombinant Human CYP1A2	1 vial	Violet	K893-100-7

V. User Supplied Reagents and Equipment:

- Multiwell fluorescence microplate reader
- Precision multi-channel pipette and reagent reservoir
- Anhydrous (reagent grade) acetonitrile and DMSO
- Black 96-well plates with flat bottom

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C and protect from light. Briefly centrifuge all small vials prior to opening. Allow the CYP1A2 Assay Buffer to warm to room temperature prior to use. Read entire protocol before performing the assay procedure.

- **3-CHC Standard:** Reconstitute in 110 µl of DMSO and vortex until fully dissolved to yield a 5 mM stock solution. The 3-CHC stock solution should be stored at -20°C and is stable for at least 3 freeze/thaw cycles.
- **CYP1A2 Inhibitor (α-naphthoflavone):** Reconstitute in 110 µl of acetonitrile and vortex until fully dissolved to yield a 1 mM stock solution. The stock solution is stable for 2 months at -20°C. To obtain a 2.5 µM working solution of α-naphthoflavone (5X final concentration), add 5 µl of the 1 mM stock solution to 1995 µl of CYP1A2 Assay Buffer. The 2.5 µM working solution should be stored at -20°C and used within one week.

- **NADPH Generating System (100X):** Reconstitute with 220 µl CYP1A2 Assay Buffer, aliquot and store at -20°C. Avoid repeated freeze/thaw cycles and keep on ice while in use.
- **β-NADP$^+$ Stock (100X):** Dissolve in 110 µl CYP1A2 Assay Buffer and vortex thoroughly to yield a 10 mM solution of NADP$^+$ (100X stock). Store at -20°C, stable for at least 3 freeze/thaw cycles.
- **CYP1A2 Substrate:** Reconstitute with 110 µl anhydrous reagent-grade acetonitrile and vortex until fully dissolved to obtain a 5 mM stock solution. Store at -20°C. Allow the vial to warm to room temperature before opening and promptly retighten cap after use to avoid absorption of airborne moisture.
- **Recombinant Human CYP1A2:** Do not reconstitute until ready to use. Reconstitute with 230 µl CYP1A2 Assay Buffer and add 20 µl of NADPH Generating System (100X). Mix thoroughly to ensure a homogenous solution (the solution will have a slightly opaque, milky appearance), aliquot and store at -80°C. Avoid repeated freeze/thaw cycles and use aliquots within one month (the Recombinant Human CYP1A2 will lose approximately 10% activity per week when stored at -80°C). Thaw aliquots rapidly at 37°C and place on ice until use (thawed aliquots should be used within 4 hours).

VII. Cytochrome P450 1A2 (CYP1A2) Activity Assay Protocol:

1. Standard Curve Preparation:

a. Dilute the 3-CHC Standard by adding 20 µl of the 5 mM solution to 480 µl CYP1A2 Assay Buffer to yield a 200 µM solution. Mix 5 µl of the 200 µM solution with 995 µl CYP1A2 Assay Buffer to generate the final 1 pmole/µl (1 µM) 3-CHC Standard. Add 0, 2, 4, 6, 8, 12, 16 and 20 µl of the 1 pmole/µl 3-CHC standard into a series of wells in an opaque 96-well plate yielding 0, 2, 4, 6, 8, 12, 16 and 20 pmole/well of 3-CHC Standard. Adjust the volume of each well to 100 µl with CYP1A2 Assay Buffer.

b. Measure fluorescence at Ex/Em = 406/468 nm. Subtract the zero standard (0 pmole/well) reading from all of the standard readings, plot the background-subtracted values and calculate the slope of the standard curve.

2. Sample and Test Compound Preparation:

a. Standardized microsomal preparations may be purchased commercially (*e.g.* donor-pooled human liver microsomes) or prepared from liver tissue or cultured cells using the Microsome Isolation Kit (Cat. #K249). Alternatively, a crude enriched lysate can be prepared: start with ~50 mg tissue or ~5 x 10^6 pelleted, pre-washed cells and homogenize in 500 µl ice-cold CYP1A2 Assay Buffer with a Dounce homogenizer (Cat. #1998 or equivalent) on ice. Incubate the homogenate on ice for 5 min. and then centrifuge at 15,000 x *g* for 15 min. in a refrigerated centrifuge at 4°C. Collect the resultant clarified supernatant for the assay in a new pre-chilled microfuge tube and store on ice until use (cell and tissue lysates can also be stored at -80°C in aliquots for future experiments).

b. If desired, CYP1A2 activity in presence of test ligands may be measured. Test ligands should be dissolved into proper solvent to produce stock solutions (see note regarding solvent effects below). For each ligand, prepare a 5X solution by diluting in CYP1A2 Assay Buffer.

Notes:

- To quantify CYP1A2 specific activity in terms of sample protein content, use the Bradford reagent (Cat. #K810) or an equivalent protein assay.
- When measuring CYP1A2 activity in presence of ligands (inhibitors or substrates), run parallel solvent control well(s) to account for additional solvent in the reaction mix. Many commonly-used organic solvents can severely impact CYP1A2 activity. Importantly, DMSO causes significant inhibition of CYP1A2 at final concentrations ≥0.25% (v/v). Our assay is designed to use acetonitrile at a final concentration of ≤1% (v/v), which has been shown to have little impact on CYP1A2 activity.

3. Reaction Preparation:

a. Prepare enough reagents for the number of reactions to be performed. For each reaction, prepare a 2X concentrated P450 reaction mix by combining 2-48 µl of sample and 2 µl of the NADPH Generating System (100X) in a 96-well plate and adjusting the final volume to 50 µl/reaction with CYP1A2 Assay Buffer. The amount of sample per reaction and the dilution factor required will vary based upon the nature of the sample. For human liver microsomes, we recommend starting with 25 µg of microsomal protein per well. For liver S9 fractions or other cellular lysates, the amount of protein required will be significantly higher. In this case, we recommend starting at 50-100 µg/well.
Note: Due to the large individual variation in CYP1A2 expression level and function, sample protein levels may need to be adjusted.

b. In addition to the test samples, prepare background control and inhibitor control (500 nM α-naphthoflavone) wells. If desired, you may also prepare CYP1A2 enzyme positive control (PC) and PC + inhibitor wells using the Recombinant Human CYP1A2 and α-naphthoflavone 2.5 µM solution (for the PC + inhibitor condition, we recommend a 1 µM final concentration of α-naphthoflavone to ensure adequate inhibition of the recombinant enzyme). Adjust the volume of test sample, inhibitor control and positive control wells to 70 µl/well with CYP1A2 Assay Buffer. For measurement of CYP1A2 activity in the presence of test ligands, replace CYP1A2 Assay Buffer with 5X concentrated test ligand solution:

	Test Sample	+ Inhibitor Control	Background	1A2 PC	PC + Inhibitor
P450 Reaction Mix (2X)	50 µl	50 µl	—	—	—
Recombinant Human CYP1A2	—	—	—	25 µl	25 µl
α-naphthoflavone 2.5 µM Solution (5X)	—	20 µl	—	—	40 µl
CYP1A2 Assay Buffer	20 µl	—	50 µl	45 µl	5 µl
Test Ligand (5X)	—	—	20 µl	—	—

c. Incubate the plate for 10-15 min at 37°C to allow the inhibitor α-naphthoflavone or any test ligands to interact with CYP1A2 in the absence of P450 catalytic turnover. During the incubation, prepare a CYP1A2 Substrate/NADP$^+$ mixture (3X) by adding 6 µl of the reconstituted 5 mM CYP1A2 Substrate stock solution and 50 µl of the reconstituted 10 mM β-NADP$^+$ Stock (100X) to 1444 µl of CYP1A2 Assay Buffer for a total volume of 1.5 ml. This preparation is sufficient for 50 reactions, but can be scaled depending upon the number of reactions to be performed.

d. Start the reaction by adding 30 µl of the CYP1A2 Substrate/NADP$^+$ (3X) mixture to each well using a multichannel pipette, yielding a final reaction volume of 100 µl/well.

Note: The Recombinant Human CYP1A2 preparation may settle and should be thoroughly mixed before dispensing.

4. **Measurement:** Immediately (within 1 min) measure the fluorescence at Ex/Em = 406/468 nm in kinetic mode for 60 min at 37°C. While the assay can be performed in either endpoint or kinetic mode, we strongly recommend reading in kinetic mode in order to ensure that the measurements recorded are within the linear range of the reaction. Ideal measurement time for the linear range may vary depending upon the content of active CYP1A2 in the sample.

   Note: Since the reaction starts immediately after the addition of the CYP1A2 Substrate/NADP$^+$ mix, it is essential to preconfigure the fluorescence microplate reader settings and use a multichannel pipette with a reagent reservoir to minimize lag time among wells.

5. **Calculation:** For each reaction well (including background and positive inhibition controls), choose two time points (T_1 and T_2) in the linear phase of the reaction progress curves, obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2) and determine the change in fluorescence over the time interval: $\Delta F = RFU_2 - RFU_1$. Subtract the ΔF value of the background control (BC) from those of the test samples (S) and 500 nM α-naphthoflavone positive inhibition control (I) to determine the background-corrected change in fluorescence intensity for each well. Calculate the specific fluorescence generated by CYP1A2 activity (denoted by C) by subtracting the positive inhibition control from each sample:

$$\boldsymbol{C_S = (\Delta F_S - \Delta F_{BC}) - (\Delta F_I - \Delta F_{BC}) = \Delta F_S - \Delta F_I}$$

Notes:

- The CYP1A2 Substrate is also metabolized by CYP2C19, necessitating the use of selective inhibitors to determine the contribution of each isozyme in heterogeneous biological samples. The concentration of α-naphthoflavone used in our assay is ≥10-fold greater than the K_i for recombinant CYP1A2, but has been shown not to affect the activity of other CYPs (Sai *et al* 2000, Xenobiotica, 30: 327-343). In human liver microsomes, this concentration typically results in 60-70% inhibition of 3-CHC formation, which represents the CYP1A2-mediated metabolic activity. In samples with significant CYP2C19 expression, the contribution of 2C19 to substrate metabolism may be tested using the selective inhibitor (+)-*N*-3-benzylnirvanol (Cat #2920) at a final concentration of 30 µM.
- In our experience, the CYP1A2 Substrate does not undergo appreciable non-enzymatic conversion to the fluorescent product. Thus, the background control (BC) well rate calculation may yield a negative value, in which case, the BC value may be ignored.

CYP1A2 metabolic activity is obtained by applying the C_S values to the 3-CHC standard curve to get B pmole of substrate metabolized to 3-CHC by CYP1A2 during the reaction time.

$$\textbf{Cytochrome P450 1A2 Specific Activity} = \frac{\boldsymbol{B}}{\boldsymbol{\Delta T \times P}} = \textbf{pmole/min/mg} = \textbf{µU/mg}$$

Where: $\mathbf{B}$ is the amount of 3-CHC produced, calculated from the standard curve (in pmole)
$\mathbf{\Delta T}$ is the linear phase reaction time $T_2 - T_1$ (in minutes)
$\mathbf{P}$ is the amount of protein in the well (in mg)

CYP1A2 Unit Definition: One unit of CYP1A2 activity is the amount of enzyme that generates 1 µmole of 3-CHC per min by hydrolysis of 1 µmole fluorogenic substrate at 37°C and pH 7.7.

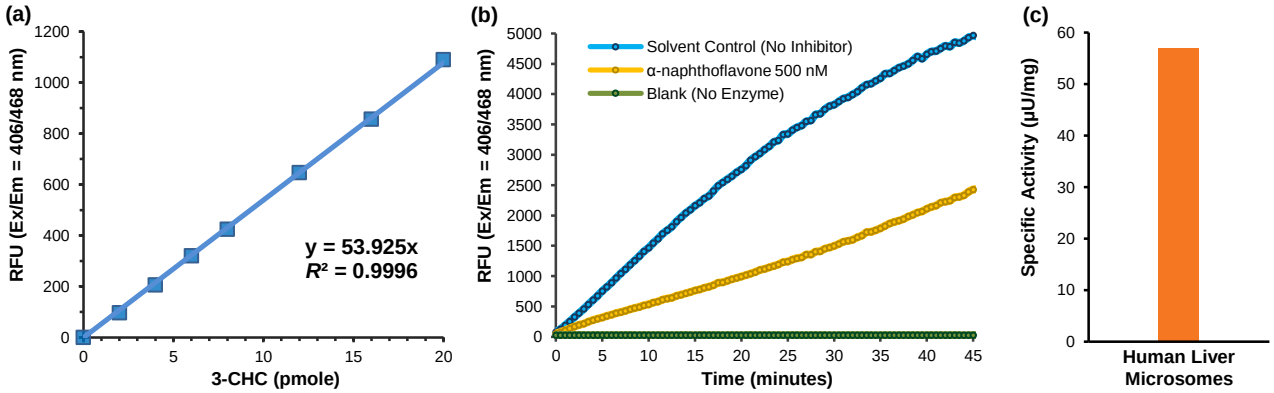


Figure: (a) 3-cyano-7-hydroxycoumarin (3-CHC) standard curve. One mole of 3-CHC corresponds to the metabolism of one mole of CYP1A2 substrate. **(b)** Reaction kinetics of fluorogenic substrate metabolism in donor-pooled human liver microsomes (0.25 mg/mL) at 37°C in the presence and absence of the CYP1A2 inhibitor α-naphthoflavone (the solvent control contained assay buffer with 0.4% acetonitrile). **(c)** Specific activity of CYP1A2 in human liver microsome sample. Assays were performed according to the kit protocol.

VIII. Related Products:

Microsome Isolation Kit (K249)
Cytochrome P450 Reductase Activity Kit (K700)
Cytochrome P450 3A4 Activity Assay Kit (K701)
Cytochrome P450 3A4 Inhibitor Screening Kit (K702)
Cytochrome P450 2D6 Activity Assay Kit (K703)
Cytochrome P450 2D6 Inhibitor Screening Kit (K704)
Cytochrome P450 2C19 Activity Assay Kit (K848)
Cytochrome P450 2C19 Inhibitor Screening Kit (K849)
Cytochrome P450 1A2 Inhibitor Screening Kit (K894)
Cytochrome b_{5A} Human Recombinant (7871)
Cytochrome P450 3A4 (CYP3A4) Human ELISA Kit (K7570)
(+)-*N*-3-benzylnirvanol (2920)

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