

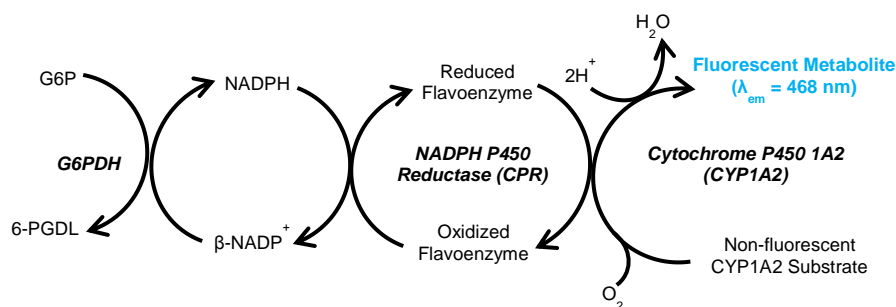
# Cytochrome P450 1A2 (CYP1A2) Inhibitor Screening Kit (Fluorometric)

(Catalog # K894-100; 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 hemoproteins 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 planar 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 methylxanthines (caffeine and theophylline), ciprofloxacin and a number of antidepressants and antipsychotics. Isoforms of the CYP1A subfamily are also involved in metabolic activation of environmental pro-carcinogens in cigarette smoke and combustion exhaust fumes. BioVision's CYP1A2 Inhibitor Screening Kit enables rapid screening of drugs and other new chemical entities (NCEs) for compound-CYP1A2 interaction in a reliable, high-throughput fluorescence-based assay. The kit provides a yeast microsomal preparation of human CYP1A2 and human cytochrome P450 reductase (CPR) enzymes. The assay utilizes a non-fluorescent CYP1A2 substrate that is converted into a highly fluorescent metabolite detected in the visible range ( $E_x/E_m = 406/468$  nm), ensuring a high signal-to-background ratio with little interference by autofluorescence. The kit contains a complete set of reagents sufficient for performing 100 reactions in a 96-well plate format.



## II. Applications:

- Rapid, high-throughput screening and characterization of drugs and novel ligands for interaction with CYP1A2.
- Development of structure-activity relationship (SAR) models to predict CYP1A2 inhibition liability of novel compounds and analogues.
- Prediction of adverse drug-drug interaction potential and bioavailability for compounds metabolized by CYP1A2.

## III. Kit Contents:

Components	K894-100	Cap Code	Part Number
CYP1A2 Assay Buffer	100 ml	NM	K894-100-1
3-CHC Standard	1 vial	Yellow	K894-100-2
CYP1A2 Inhibitor ( $\alpha$ -naphthoflavone)	1 vial	Blue	K894-100-3
NADPH Generating System (100X)	1 vial	Green	K894-100-4
$\beta$ -NADP <sup>+</sup> Stock (100X)	1 vial	Amber	K894-100-5
CYP1A2 Substrate	1 vial	Red	K894-100-6
Recombinant Human CYP1A2	2 vials	Violet	K894-100-7

## IV. User Supplied Reagents and Equipment:

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

## V. Storage Conditions and Reagent Preparation:

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

- **3-CHC Standard:** Reconstitute in 110  $\mu$ l 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 ( $\alpha$ -naphthoflavone):** Reconstitute in 110  $\mu$ l 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 30  $\mu$ M working solution of  $\alpha$ -naphthoflavone (5X final concentration), add 30  $\mu$ l of the 1 mM stock solution to 970  $\mu$ l of CYP1A2 Assay Buffer. The 30  $\mu$ M working solution should be stored at -20°C and used within one week.
- **NADPH Generating System (100X):** Reconstitute with 110  $\mu$ l CYP1A2 Assay Buffer, aliquot and store at -20°C. Avoid repeated freeze/thaw cycles. Keep on ice while in use.
- **$\beta$ -NADP<sup>+</sup> Stock (100X):** Dissolve in 110  $\mu$ l CYP1A2 Assay Buffer and vortex thoroughly (100X stock). Store at -20°C, stable for at least 3 freeze/thaw cycles.



- **CYP1A2 Substrate:** Reconstitute with 110  $\mu$ l anhydrous HPLC-grade acetonitrile and vortex until fully dissolved to obtain a 5 mM stock solution. Store at  $-20^{\circ}\text{C}$ . When using the CYP1A2 Substrate stock solution, allow the vial to warm to RT before opening and promptly retighten cap after use to avoid absorption of airborne moisture.
- **Recombinant Human CYP1A2:** The Recombinant Human CYP1A2 should be reconstituted immediately before use as directed in Section VI.2 below. Each vial is sufficient for preparation of 50 reactions in a 96-well plate format.

## VI. Cytochrome P450 1A2 (CYP1A2) Inhibitor Screening Protocol:

### 1. Standard Curve Preparation:

- a. Dilute the 3-CHC Standard by adding 20  $\mu$ l of the 5 mM solution to 480  $\mu$ l CYP1A2 Assay Buffer to yield a 200  $\mu$ M solution. Mix 5  $\mu$ l of the 200  $\mu$ M solution with 995  $\mu$ l CYP1A2 Assay Buffer to generate the final 1 pmole/ $\mu$ l (1  $\mu$ M) 3-CHC Standard. Add 0, 2, 4, 6, 8, 12, 16 and 20  $\mu$ l of the 1 pmole/ $\mu$ l 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 3-CHC Standard. Adjust the volume of each well to 100  $\mu$ l 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. Test Compound and CYP1A2 Enzyme Preparation:

- a. Dissolve test compounds into proper solvent to produce stock solutions (see note regarding solvent effects below). For each test compound, prepare a 5X solution of each desired test concentration by diluting in CYP1A2 Assay Buffer. To determine  $\text{IC}_{50}$  values for test compounds, 5X test compound solutions should be prepared in a range of concentrations in order to generate a multi-point dose-response curve. It is also possible to perform a cursory initial screen of a large number of test compounds by observing the percent inhibition at a single fixed concentration of each test compound. In this case, we recommend a final test compound concentration of 3  $\mu$ M, for which 15  $\mu$ M solutions (5X final concentration) should be prepared.
- b. Prepare the Recombinant Human CYP1A2 stock (2X) by reconstituting with 1 ml of CYP1A2 Assay Buffer. Mix contents thoroughly by vortexing to obtain a homogeneous solution (the solution will have a slightly opaque, milky appearance) and transfer the solution to a 15 ml conical tube. Bring the volume up to 2450  $\mu$ l with CYP1A2 Assay Buffer and add 50  $\mu$ l of the NADPH Generating System (100X) for a final total volume of 2.5 ml. The CYP1A2 stock is stable for up to 4 hours at room temperature or one day if kept on ice. In order to minimize enzyme instability, we do not recommend long term storage of the reconstituted enzyme system mix.

**Note:** Many commonly-used organic solvents can severely impact CYP1A2 activity. Importantly, DMSO and methanol cause significant inhibition of CYP1A2 at final concentrations of  $\geq 0.25\%$  (v/v). We recommend using acetonitrile (final concentration  $\leq 1\%$ ; the CYP1A2 Substrate contributes 0.08% acetonitrile to the reaction volume) to dissolve any test ligands, which has been shown to have the least impact on CYP activity. We recommend preparing a parallel solvent control well with the same final concentration of solvent used to solubilize the test ligands, particularly if using a solvent other than acetonitrile.

### 3. Reaction Preparation:

- a. Prepare reaction wells containing test compounds, corresponding no inhibitor controls (which may also serve as a solvent control), background controls and (if desired) a positive inhibition control using 30  $\mu$ M  $\alpha$ -naphthoflavone (5X solution):

	No Inhibitor	+ Test Compound	Background Control	Positive Inhibition Control
CYP1A2 Stock (2X)	50 $\mu$ l	50 $\mu$ l	—	50 $\mu$ l
Test Compound Solution (5X)	—	20 $\mu$ l	—	—
$\alpha$ -naphthoflavone 30 $\mu$ M Solution (5X)	—	—	—	20 $\mu$ l
CYP1A2 Assay Buffer (+5X Solvent)	20 $\mu$ l	—	70 $\mu$ l	—

- b. Incubate the plate for 10-15 min at  $37^{\circ}\text{C}$  to allow the test compounds to permeate the microsomal membranes and interact with CYP1A2 in the absence of P450 catalytic turnover. During the incubation, prepare a CYP1A2 Substrate/NADP<sup>+</sup> mixture (3X) by adding 4  $\mu$ l of the reconstituted 5 mM CYP1A2 Substrate stock solution and 50  $\mu$ l of the reconstituted 10 mM  $\beta$ -NADP<sup>+</sup> stock (100X) to 1446  $\mu$ l of CYP1A2 Assay Buffer for a total volume of 1.5 ml. This preparation is sufficient for 50 reaction wells, but can be adjusted depending upon the number of reactions to be performed.
- c. Start the reaction by adding 30  $\mu$ l of the CYP1A2 Substrate/NADP<sup>+</sup> (3X) mixture to each well using a multichannel pipette, yielding a final reaction volume of 100  $\mu$ l/well.

#### Notes:

- To ensure maximal signal intensity, both the pre-incubation period and the P450 reaction itself should be performed at  $37^{\circ}\text{C}$ .
- The microsomal membranes in the recombinant human CYP1A2 stock may settle at the bottom of the tube over time, so it may be necessary to re-mix to ensure a homogenous solution before dispensing.
- For no inhibitor/solvent control condition, prepare a small aliquot of CYP1A2 Assay Buffer containing the organic solvent used to dissolve the test compounds at 5X final concentration.
- During the pre-incubation period, the plate can be pre-read to determine if any test compounds are intrinsically fluorescent.
- The suggested starting point for the final concentration of CYP1A2 Substrate is 4  $\mu$ M, which is approximately equal to the  $K_m$  for the recombinant CYP1A2 enzyme. This can be optimized by the user depending on the inhibitory potency of their test compounds and the mechanism of inhibition.

4. **Measurement:** Immediately (within 1 min) measure the fluorescence at Ex/Em = 406/468 nm in kinetic mode for 60 min. 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 exact reaction temperature and experimental conditions.

**Note:** Since the reaction starts immediately after the addition of the CYP1A2 Substrate/NADP<sup>+</sup> 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.

