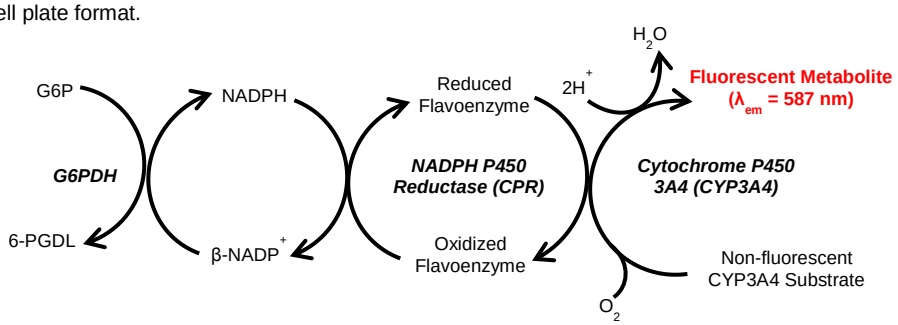
Gentaur Europe BVBA Voortstraat 49, 1910 Kampenhout BELGIUM
Tel 0032 16 58 90 45 info@gentaur.com

Cytochrome P450 3A4 (CYP3A4) Inhibitor Screening Kit (Fluorometric)

(Catalog # K702-200; 200 Reactions; Store at -20°C)

I. Introduction:

Cytochrome P450 3A4 (CYP3A4, EC 1.14.13.157) 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 transformed to more hydrophilic products to facilitate excretion from the body. CYP3A4 is expressed in high levels in the liver and intestines, where it catalyzes oxidation of an extraordinarily wide variety of structurally distinct ligands. More than half of all small molecule drugs commonly used by humans are metabolized by CYP3A4 and inhibition of CYP3A4-mediated metabolism is a common cause of adverse drug/drug and drug/food interactions and toxicity. In addition, for drugs whose pharmacological activity requires metabolism from a pro-drug form, CYP3A4 inhibition can lead to decreased drug efficacy. BioVision's CYP3A4 Inhibitor Screening Kit enables rapid screening of drugs and other new chemical entities (NCEs) for compound-CYP3A4 interaction in a reliable, high-throughput fluorescence-based assay. The kit provides a yeast microsomal preparation of human CYP3A4 and cytochrome P450 reductase (CPR) enzymes. The assay utilizes a non-fluorescent CYP3A4 substrate that is converted into a highly fluorescent metabolite detected in the visible range (Ex/Em = 535/587 nm), ensuring a high signal-to-background ratio with little interference by autofluorescence. The concentration of fluorogenic substrate used for screening is roughly equivalent to its K_m for CYP3A4, facilitating detection of weak competitive inhibitors. The kit contains a complete set of reagents sufficient for performing 200 reactions in a 96-well plate format.



II. Applications:

- Rapid, high-throughput screening and characterization of drugs and novel ligands for interaction with CYP3A4.
- Development of structure-activity relationship (SAR) models to predict CYP3A4 inhibition liability of novel compounds and analogues.
- Prediction of adverse drug-drug interaction potential and bioavailability for compounds metabolized by CYP3A4.

III. Kit Contents:

Components	K702-200	Cap Code	Part Number
CYP3A4 Assay Buffer	100 ml	NM	K702-200-1
Resorufin Standard (5 mM in DMSO)	50 µl	Yellow	K702-200-2
CYP3A4 Inhibitor (Ketoconazole)	1 vial	Amber	K702-200-3
NADPH Generating System (100X)	1 vial	Green	K702-200-4
β-NADP$^+$ Stock (100X)	1 vial	Blue	K702-200-5
CYP3A4 Substrate	1 vial	Red	K702-200-6
Recombinant Human CYP3A4	2 vials	Violet	K702-200-7

IV. User Supplied Reagents and Equipment:

- Multiwell fluorescence microplate reader
- Precision multi-channel pipette and reagent reservoir
- Anhydrous (reagent grade) acetonitrile
- Opaque 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 CYP3A4 Assay Buffer to warm to room temperature (RT) prior to use. Read entire protocol before performing the assay procedure.

- **Resorufin Standard:** Warm the Resorufin Standard to RT and vortex prior to use. The Resorufin Standard stock solution should be stored at -20°C and is stable for at least 3 freeze/thaw cycles.
- **CYP3A4 Inhibitor (Ketoconazole):** The inhibitor Ketoconazoleis provided as a dried film. Reconstitute in 220 µl of acetonitrile and vortex until fully dissolved to yield a 5 mM stock solution. The stock solution is stable for 2 months at -20°C. To obtain a 150 µM working solution of Ketoconazole (5X final concentration), add 30 µl of the 5 mM Ketoconazole stock solution to 970 µl of CYP3A4 Assay Buffer. Store the resulting 5X Ketoconazole working solution at -20°C and use within one week.
- **NADPH Generating System (100X):** Reconstitute with 220 µl CYP3A4 Assay Buffer, aliquot and store at -20°C. Avoid repeated freeze/thaw cycles. Keep on ice while in use.
- **β-NADP$^+$ Stock (100X):** Dissolve in 220 µl CYP3A4 Assay Buffer and vortex thoroughly to yield a 10 mM stock solution of NADP$^+$ (100X final concentration). Store at -20°C, stable for at least 3 freeze/thaw cycles.

- **CYP3A4 Substrate:** Reconstitute with 220 µl dry HPLC-grade acetonitrile and vortex until fully dissolved to obtain a 2 mM stock solution. Store at -20°C. When using the CYP3A4 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 CYP3A4:** The Recombinant Human CYP3A4 should be reconstituted immediately before use as directed in Section VI.2 below. Each vial is sufficient for one 96-plate.

VI. Cytochrome P450 3A4 (CYP3A4) Inhibitor Screening Kit Protocol:

1. Standard Curve Preparation:

a. Dilute the Resorufin Standard (5 mM stock solution) by adding 2 µl of the 5 mM solution to 998 µl CYP3A4 Assay Buffer to generate a 10 pmole/µl (10 µM) solution of resorufin. Mix 50 µl of the 10 pmole/ µl resorufin solution with 450 µl CYP3A4 Assay Buffer to generate the final 1 pmol/µl (1 µM) resorufin standard. Add 0, 4, 8, 12, 16, 20, 30 and 40 µl of the 1 pmole/µl resorufin standard into a series of wells in an opaque 96-well plate. Adjust the volume of each well to 100 µl with CYP Assay Buffer, yielding a standard curve with 0, 4, 8, 12, 16, 20, 30 and 40 pmole/well of resorufin.

b. Measure fluorescence at Ex/Em = 535/587 nm. Subtract the blank reading (0 nmole/well) from all of the resorufin standard readings, plot the background-subtracted values and calculate the slope of the standard curve.

2. Test Compound and CYP3A4 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 CYP3A4 Assay Buffer. To determine 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 µM, for which 15 µM solutions (5X final concentration) should be prepared.

b. Prepare the Recombinant Human CYP3A4 stock (2X) by reconstituting with 1 ml of CYP3A4 Assay Buffer. Mix contents thoroughly by vortexing to obtain a homogeneous solution and transfer the solution to a 15 ml conical tube. Bring the volume up to 4.9 ml with CYP3A4 Assay Buffer and add 100 µl of the NADPH Generating System (100X) for a final total volume of 5 ml. The CYP3A4 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 CYP3A4 activity. Importantly, DMSO causes significant inhibition of CYP3A4 at final concentrations of ≥0.1% (v/v). We recommend using acetonitrile (final concentration ≤1%; the CYP3A4 substrate contributes 0.1% acetonitrile to the reaction volume when used at 2 µM) to dissolve any test ligands, which has been shown to have the least impact on CYP3A4 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 (no enzyme) and (if desired) a positive inhibition control using 150 µM Ketoconazole (5X solution):

	No Inhibitor	+ Test Compound	Background Control	Positive Inhibition Control
CYP3A4 Enzyme Stock (2X)	50 µl	50 µl	—	50 µl
Test Compound Solution (5X)	—	20 µl	—	—
Ketoconazole 150 µM Solution (5X)	—	—	—	20 µl
CYP3A4 Assay Buffer (+5X Solvent)	20 µl	—	70 µl	—

b. Incubate the plate for 5-10 min to allow the test compounds to permeate the microsomal membranes and interact with CYP3A4 in the absence of P450 catalytic turnover. During the incubation, prepare a CYP3A4 Substrate/$NADP^+$ mixture (3X) by adding 10 µl of the reconstituted 2 mM CYP3A4 Substrate stock solution and 100 µl of the reconstituted β-$NADP^+$ Stock (100X) to 2890 µl of CYP3A4 Assay Buffer for a total volume of 3 ml. This preparation is sufficient for one 96-well plate.

c. Start the reaction by adding 30 µl of the CYP3A4 Substrate/$NADP^+$ (3X) mixture to each well using a multichannel pipette, yielding a final reaction volume of 100 µl/well.

Notes:

- The pre-incubation and P450 reaction may be performed at any temperature between RT and 37°C. In our experience, performing the assay at RT rather than 37°C gives a longer linear phase with little loss of signal intensity.
- The microsomal membranes in the recombinant human CYP3A4 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 CYP3A4 Assay Buffer containing the organic solvent used to dissolve the test compounds at 5X final concentration.
- During the pre-incubation period, you may wish to pre-read the plate to determine if any test compounds are intrinsically fluorescent.
- The suggested starting point for the final concentration of CYP3A4 Substrate is 2 µM, which is ~K_m for the recombinant CYP3A4 enzyme.

4. Measurement:

Immediately (within 1 min) measure the fluorescence at Ex/Em = 535/587 nm in kinetic mode for 30-45 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 reaction temperature and experimental conditions.

Note: Since the reaction starts immediately after the addition of the CYP3A4 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 no inhibitor controls), choose two time points (T_1 and T_2) in the linear phase of the reaction progress curve, obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2) and determine the rate of change in fluorescence over the time interval: $\Delta F/\Delta T = (RFU_2 - RFU_1) / (T_2 - T_1)$. Subtract the rate of the no enzyme/background control (BC) well from the rates of each of the no inhibitor/solvent control (SC) and test compound (TC) wells to determine background-corrected reaction rates (denoted by R) for each well:

$$R_{SC} = \frac{\Delta F_{SC}}{\Delta T_{SC}} - \frac{\Delta F_{BC}}{\Delta T_{BC}} \text{ and } R_{TC} = \frac{\Delta F_{TC}}{\Delta T_{TC}} - \frac{\Delta F_{BC}}{\Delta T_{BC}}$$

Calculate the percent inhibition due to the test ligand or positive inhibition control using the following equation:

$$\% \text{ Relative Inhibition} = \frac{R_{SC} - R_{TC}}{R_{SC}} \times 100\%$$

Note: If desired, reaction rate calculations can also be expressed in terms of pmole resorufin formed per unit time per unit amount of protein by interpolation from the standard curve. Each well will contain a total of 50 µg of protein when the recombinant human CYP3A4 is used at the proportions suggested in the kit protocol.

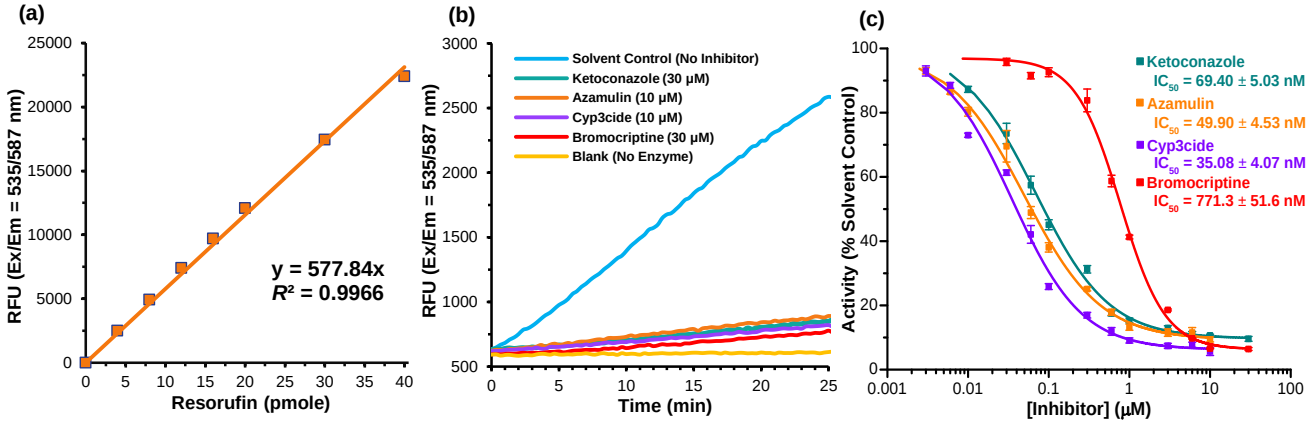


Figure: (a) Resorufin standard curve. One mole of resorufin corresponds to the metabolism of one mole of CYP3A4 substrate. **(b)** Reaction kinetics of recombinant human CYP3A4 enzyme at 25°C in the presence and absence of the indicated CYP3A4 inhibitors (the solvent control reaction contained a final concentration of 0.7% acetonitrile). **(c)** Dose-response curves for various CYP3A4 inhibitors of differing structural and mechanistic classes: the canonical competitive CYP3A4 inhibitor Ketoconazole, the selective mechanism-based CYP3A4 inhibitors Azamulin (Cat #2915) and cyp3cide (PF-4981517), and bromocriptine, a CYP3A4 substrate that acts as a weak enzyme inhibitor. For dose-response curves, percent activity was calculated for each concentration of inhibitor by comparison to activity of reactions containing no inhibitor. For each CYP3A4 inhibitor, IC_{50} values were derived by 4-parameter logistic curve fitting and represent the mean ± SEM of at least three replicate experiments. Assays were performed according to the kit protocol.

VII. RELATED PRODUCTS:

Microsome Isolation Kit (K249)
Cytochrome P450 Reductase Activity Kit (K700)
Cytochrome P450 3A4 Activity Assay Kit (K701)
Cytochrome P450 3A4 (CYP3A4) Mouse ELISA Kit (K7570)
Cytochrome P450 3A4 (CYP3A4) Mouse ELISA Kit (K7571)
Cytochrome b_{5A} Human Recombinant (7871)
Itraconazole (1987)
Progesterone (2913)
Rosiglitazone (1559)
Azamulin (2915)

FOR RESEARCH USE ONLY! Not to be used on humans.