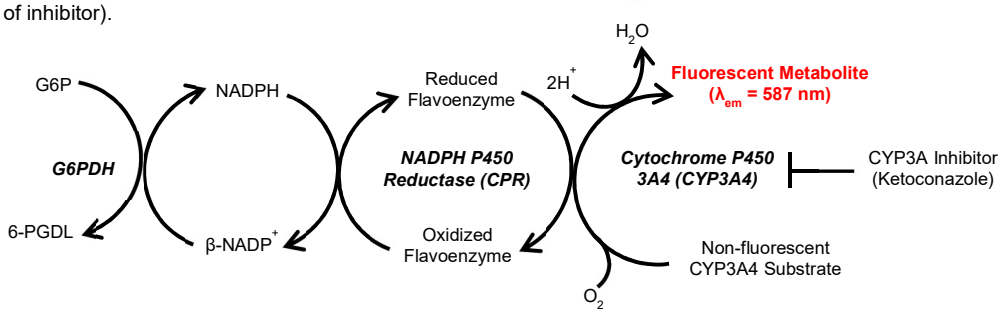
Cytochrome P450 3A4 (CYP3A4) Activity Assay Kit (Fluorometric)

(Catalog # BN00925; 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. 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. Assay Genie's CYP3A4 Activity Assay Kit enables rapid measurement of native or recombinant CYP3A4 activity in biological samples such as liver microsomes. 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. CYP3A4 specific activity is calculated by running parallel reactions in the presence and absence of the potent inhibitor Ketoconazole and subtracting any residual activity detected with the inhibitor present. The kit contains a complete set of reagents sufficient for performing 100 sets of paired reactions (in the presence and absence of inhibitor).



II. Applications:

- Rapid assessment of native/recombinant CYP3A4 activity in fractions prepared from tissues and cells.
- Screening of drugs and novel ligands for interaction with native/recombinant CYP3A4.

III. Sample Type:

- Human liver microsomes and liver S9 fractions
- Lysates of tissues and cultured cells, primary hepatocytes
- Heterologously expressed recombinant CYP3A4 preparations

IV. Kit Contents:

Components	BN00925	Cap Code	Part Number
CYP3A4 Assay Buffer	100 ml	NM	BN00925-1
Resorufin Standard (5 mM in DMSO)	50 µl	Yellow	BN00925-2
CYP3A4 Inhibitor (Ketoconazole)	1 vial	Amber	BN00925-3
NADPH Generating System (100X)	1 vial	Green	BN00925-4
β-NADP+ Stock (100X)	1 vial	Blue	BN00925-5
CYP3A4 Substrate	1 vial	Red	BN00925-6
Recombinant Human CYP3A4	1 vial	Violet	BN00925-7

V. 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

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 CYP3A4 Assay Buffer to warm to room temperature prior to use. Read entire protocol before performing the assay procedure.

- **Resorufin Standard:** Warm the Resorufin Standard to room temperature and vortex prior to use. Store at -20°C, stable for at least 3 freeze/thaw cycles.
- **CYP3A4 Inhibitor (Ketoconazole):** The inhibitor is provided as a dried film. Reconstitute in 220 µl of dry reagent-grade acetonitrile and vortex until fully dissolved to yield a 5 mM stock solution (stable for up to 2 months at -20°C). To obtain a 150 µM working solution of Ketoconazole (5X final concentration) add 30 µl of the 5 mM stock solution to 970 µl of CYP3A4 Assay Buffer. Store the 5X Ketoconazole working solution at -20°C and use within one week.
- **NADPH Generating System (100X):** Reconstitute with 440 µl CYP3A4 Assay Buffer, aliquot and store at -20°C. Avoid repeated freeze/thaw cycles and keep on ice while in use.

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- **β-NADP$^+$ Stock (100X):** Dissolve in 220 µl CYP3A4 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.
- **CYP3A4 Substrate:** Reconstitute with 220 µl dry reagent-grade acetonitrile and vortex until fully dissolved to obtain a 2 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 CYP3A4:** Do not reconstitute until ready to use. Reconstitute with 460 µl CYP3A4 Assay Buffer and add 40 µl of NADPH Generating System (100X). Mix thoroughly to ensure a homogenous solution, aliquot and store at -80°C. Avoid repeated freeze/thaw cycles and use aliquots within one month (the Recombinant Human CYP3A4 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 3A4 (CYP3A4) Activity Assay 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 pmol/µl resorufin standard solution 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. 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. 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 CYP3A4 Assay Buffer with a Dounce homogenizer 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, CYP3A4 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 CYP3A4 Assay Buffer.

Notes:

- To quantify CYP3A4 specific activity in terms of sample protein content, use the Bradford reagent or an equivalent protein assay.
- In case the sample contains CYP3A5/CYP3A7 isoforms that can contribute to CYP3A activity, Azamulin can be used since it is 15- and 13-fold more potent as an inhibitor of CYP3A4 as compared to CYP3A5 and CYP3A7, respectively (Stresser *et al*. 2004, DMD, 32: 105-112).
- When measuring CYP3A4 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 CYP3A4 activity. Importantly, DMSO causes significant inhibition of CYP3A4 at final concentrations ≥0.1% (v/v). Our assay is designed to use acetonitrile at a final concentration of ≤1%, which has been shown to have little impact on CYP3A4 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 CYP3A4 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 that the 2X reaction mix contain 0.1-0.2 mg/ml microsomal protein (yielding a final concentration of 0.05-0.1 mg/ml or 5-10 µg of microsomes per well). For liver S9 fractions or other cellular lysates, the amount of protein required will be significantly higher (we recommend starting at 1-2 mg/ml protein or 50-100 µg/well).

b. In addition to the test samples, prepare background control (no enzyme) and inhibitor control (30 µM Ketoconazole) wells. If desired, you may also prepare CYP3A4 enzyme positive control wells using the Recombinant Human CYP3A4. Adjust the volume of test sample, inhibitor control and positive control wells to 70 µl/well with CYP3A4 Assay Buffer. For measurement of CYP3A4 activity in the presence of test ligands, replace CYP3A4 Assay Buffer with 5X concentrated test ligand solution:

	Test Sample	+ Inhibitor Control	Background	Positive Control
P450 Reaction Mix (2X)	50 µl	50 µl	—	—
Recombinant Human CYP3A4	—	—	—	25 µl
Ketoconazole 150 µM Solution (5X)	—	20 µl	—	—
CYP3A4 Assay Buffer (or Test Ligand 5X)	20 µl	—	70 µl	45 µl

c. Incubate the plate for 5-10 min at 37°C to allow the inhibitor Ketoconazole or any test ligands to 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 100 reactions, but can be scaled depending upon the number of reactions to be performed.

d. 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.

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Notes:

- The suggested final concentration of CYP3A4 Substrate is 2 µM, which is ~K_m for the recombinant CYP3A4.
- The Recombinant Human CYP3A4 preparation may settle and should be thoroughly mixed before dispensing.

4. **Measurement:** Immediately (within 1 min) measure the fluorescence at Ex/Em = 535/587 nm in kinetic mode for 30-45 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 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 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 30 µM Ketoconazole positive inhibition control (K) to determine the background-corrected change in fluorescence intensity for each well. Calculate the specific fluorescence generated by CYP3A4 activity (denoted by C) by subtracting the background-corrected positive inhibition control from each sample:

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

CYP3A4 metabolic activity is obtained by applying the C_S values to the resorufin standard curve to get B pmole of substrate metabolized to resorufin by CYP3A4 during the reaction time.

$$\textbf{Cytochrome P450 3A4 Specific Activity} = \frac{\boldsymbol{B}}{\boldsymbol{\Delta T \times P}} = \textbf{pmole/min/mg} = \textbf{µU/mg}$$

Where: $\mathbf{B}$ is the amount of resorufin 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 sample (in mg)

CYP3A4 Unit Definition: One unit of CYP3A4 activity is the amount of enzyme that generates 1 µmole of resorufin per min by hydrolysis of 1 µmole fluorogenic substrate at 37°C and pH 7.7.

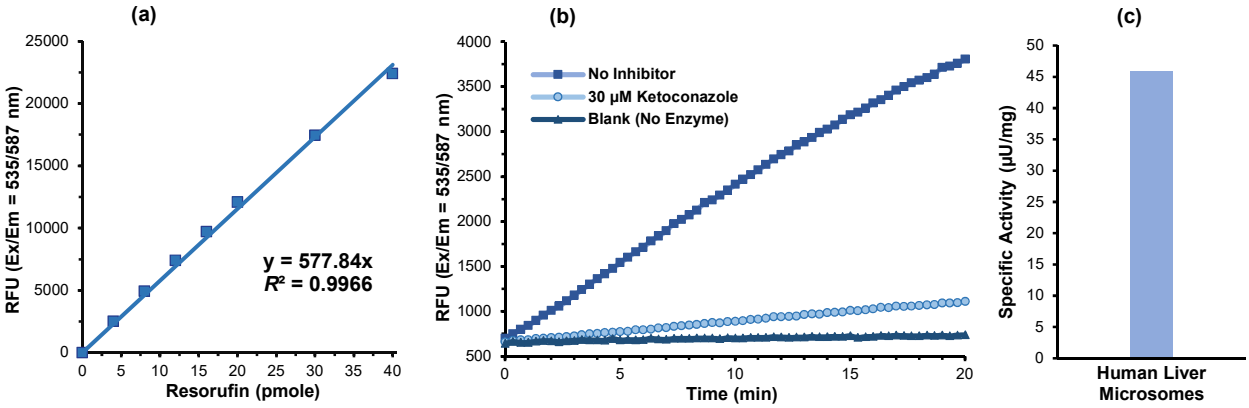

Figure: (a) Resorufin standard curve. One mole of resorufin corresponds to the metabolism of one mole of CYP3A4 substrate. **(b)** Reaction kinetics of fluorogenic substrate metabolism in human liver microsomes (0.05 mg/mL) at 37°C in the presence and absence of the CYP3A4 inhibitor Ketoconazole (the no inhibitor reaction contained a final concentration of 0.6% acetonitrile). **(c)** Specific activity of CYP3A4 in pooled human liver microsomes (0.05 mg/mL). Assays were performed according to the kit protocol.

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