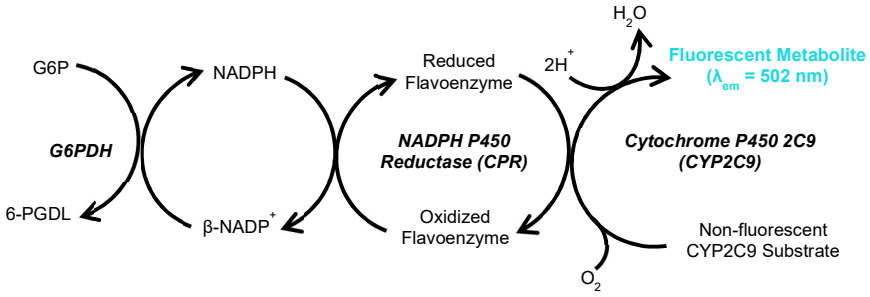
Cytochrome P450 2C9 (CYP2C9) Inhibitor Screening Kit (Fluorometric) (#BN01070)

(Catalog # BN01070; 100 Reactions; Store at -20°C)

I. Introduction:

Cytochrome P450 2C9 (CYP2C9, 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. CYP2C9 is primarily expressed in liver and catalyzes oxidation of small, weakly acidic or hydrophobic molecules containing an aromatic moiety and at least one hydrogen bond donor. Isoforms of the CYP2C subfamily are responsible for metabolism of nearly 20% of all small molecule drugs commonly used by humans. The human CYP2C9 gene is highly polymorphic and CYP2C9 single nucleotide polymorphisms (SNPs) have been implicated in clinical drug/drug interactions involving widely-prescribed drugs with narrow therapeutic indices such as warfarin, phenytoin and indomethacin. In addition, for drugs whose pharmacological activity requires metabolism from a pro-drug form (such as the antihypertensive drug losartan), CYP2C9 inhibition or allelic deficiency can lead to decreased drug efficacy. Assay Genie's CYP2C9 Inhibitor Screening Kit enables rapid screening of drugs and other new chemical entities (NCEs) for compound-CYP2C9 interaction in a reliable, high-throughput fluorescence-based assay. The kit provides a yeast microsomal preparation of human CYP2C9 and human cytochrome P450 reductase (CPR) enzymes. The assay utilizes a non-fluorescent CYP2C9 substrate that is converted into a highly fluorescent metabolite detected in the visible range (Ex/Em = 415/502 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 CYP2C9.
- Development of structure-activity relationship (SAR) models to predict CYP2C9 inhibition liability of novel compounds and analogues.
- Prediction of adverse drug-drug interaction potential and bioavailability for compounds metabolized by CYP2C9.

III. Kit Contents:

Components	BN01070	Cap Code	Part Number
CYP2C9 Assay Buffer	100 ml	NM	BN01070-1
7-HFC Standard	1 vial	Red	BN01070-2
CYP2C9 Inhibitor (Sulfaphenazole)	1 vial	Amber	BN01070-3
NADPH Generating System (100X)	1 vial	Green	BN01070-4
β-NADP+ Stock (100X)	1 vial	Blue	BN01070-5
CYP2C9 Substrate	1 vial	Yellow	BN01070-6
Recombinant Human CYP2C9	2 vials	Violet	BN01070-7

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 CYP2C9 Assay Buffer to warm to room temperature (RT) prior to use. Read entire protocol before performing the assay procedure.

- **7-HFC Standard:** Reconstitute in 110 µl of DMSO and vortex until fully dissolved to yield a 5 mM stock solution. The 7-HFC stock solution should be stored at -20°C and is stable for at least 3 freeze/thaw cycles.
- **CYP2C9 Inhibitor (Sulfaphenazole):** Reconstitute in 55 µl of acetonitrile and vortex until fully dissolved to yield a 20 mM stock solution. The stock solution is stable for 2 months at -20°C. To obtain a 300 µM working solution of sulfaphenazole (5X final concentration), add 15 µl of the 20 mM stock solution to 985 µl of CYP2C9 Assay Buffer. The 300 µM working solution should be stored at -20°C and used within one month.
- **NADPH Generating System (100X):** Reconstitute with 110 µl CYP2C9 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 110 µl CYP2C9 Assay Buffer and vortex thoroughly (100X stock). Store at -20°C, stable for at least 3 freeze/thaw cycles.
- **CYP2C9 Substrate:** Reconstitute with 55 µl anhydrous HPLC-grade acetonitrile and vortex until fully dissolved to obtain a 10 mM stock solution. Store at -20°C. When using the CYP2C9 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 CYP2C9:** The Recombinant Human CYP2C9 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 2C9 (CYP2C9) Inhibitor Screening Protocol:

1. Standard Curve Preparation:

a. Dilute the 7-HFC Standard by adding 20 µl of the 5 mM solution to 480 µl CYP2C9 Assay Buffer to yield a 200 µM solution. Mix 5 µl of the 200 µM solution with 995 µl CYP2C9 Assay Buffer to generate the final 1 pmole/µl (1 µM) 7-HFC Standard. Add 0, 2, 4, 6, 8, 12, 16 and 20 µl of the 1 pmole/µl 7-HFC standard into a series of wells in an opaque 96-well plate, yielding 0, 2, 4, 6, 8, 12, 16 and 20 pmole/well 7-HFC Standard. Adjust the volume of each well to 100 µl with CYP2C9 Assay Buffer.

b. Measure fluorescence at Ex/Em = 415/502 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 CYP2C9 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 CYP2C9 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 CYP2C9 stock (2X) by reconstituting with 1 ml of CYP2C9 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 µl with CYP2C9 Assay Buffer and add 50 µl of the NADPH Generating System (100X) for a final total volume of 2.5 ml. The CYP2C9 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 CYP2C9 activity. Importantly, DMSO causes significant inhibition of CYP2C9 at final concentrations of ≥0.25% (v/v). We recommend using acetonitrile at a final concentration ≤1% (the CYP2C9 Substrate contributes 0.4% 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 (SC) well with the same final concentration of solvent used to solubilize the test ligands, particularly if using a solvent other than acetonitrile and use this well to determine 100% activity if significantly different from No Inhibitor well(s) in 3.a. below.

3. Reaction Preparation:

a. Prepare reaction wells containing test compounds and corresponding no inhibitor controls (which may also serve as a solvent control), as well as a background control (which contains no fluorogenic CYP2C9 Substrate) and (if desired) a positive inhibition control using 300 µM sulfaphenazole (5X solution, 60 µM final concentration):

	No Inhibitor	+ Test Compound	Background Control	Positive Inhibition Control
CYP2C9 Stock (2X)	50 µl	50 µl	50 µl	50 µl
Test Compound Solution (5X)	—	20 µl	—	—
Sulfaphenazole 300 µM Solution (5X)	—	—	—	20 µl
CYP2C9 Assay Buffer (+5X Solvent)	20 µl	—	50 µl	—

b. Incubate the plate for 15-20 min at 37°C to allow the test compounds to permeate the microsomal membranes and interact with CYP2C9 in the absence of P450 catalytic turnover. During the incubation, prepare a CYP2C9 Substrate/$NADP^+$ mixture (3X) by adding 20 µl of the reconstituted 10 mM CYP2C9 Substrate stock solution and 50 µl of the reconstituted 10 mM β-$NADP^+$ stock (100X) to 1430 µl of CYP2C9 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 µl of the CYP2C9 Substrate/$NADP^+$ (3X) mixture to each well (aside from the background control) using a multichannel pipette, yielding a final reaction volume of 100 µl/well.

Notes:

- To ensure maximal signal intensity, both the pre-incubation period and the P450 reaction itself should be performed at 37°C.
- The microsomal membranes in the recombinant human CYP2C9 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 CYP2C9 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 CYP2C9 Substrate is 40 µM, which is approximately equal to the K_m for the recombinant CYP2C9 enzyme. This can be optimized by the user depending on the inhibitory potency of their test compounds and the mechanism of inhibition.
- The recombinant CYP2C9 enzyme may exhibit a slight increase in fluorescence, even in the absence of fluorogenic CYP2C9 Substrate. Hence, we recommend preparing a “no substrate” well as the background control. In our experience, the CYP2C9 Substrate does not undergo any appreciable non-enzymatic conversion to the fluorescent product, obviating the need for a “no enzyme” background control.

4. Measurement:

Immediately (within 1 min) measure the fluorescence at Ex/Em = 415/502 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 CYP2C9 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 $\Delta F = (RFU_2 - RFU_1)$ and ΔT ($T_2 - T_1$). Calculate the rate of change in fluorescence over time according to the equation below. Subtract the rate of the no substrate/background control (BC) well from the rates of each of the no inhibitor/solvent control (R_{SC}) and test compound (R_{TC}) wells to determine background-corrected reaction rates (denoted by R) for each well:

$$R = \frac{\Delta F - \Delta F_{BC}}{\Delta T}$$

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

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

Notes:

- If desired, reaction rate calculations can also be expressed in terms of pmoles of 7-HFC formed per unit time per unit amount of protein by interpolation from the standard curve. Each well will contain a total of 100 µg of protein when the recombinant human CYP2C9 is used at the proportions suggested in the kit protocol.

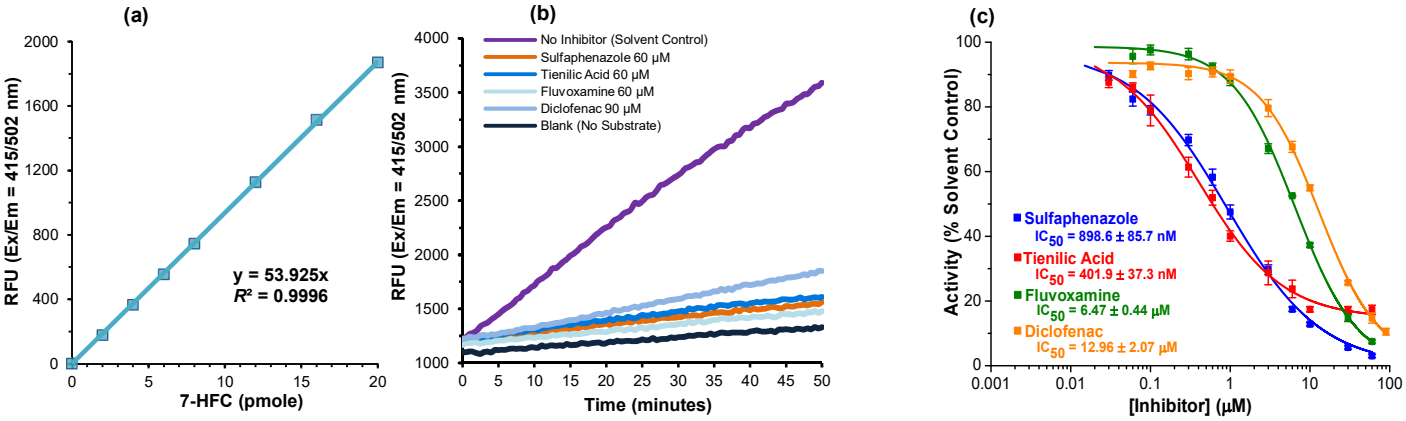


Figure: (a) 7-hydroxy-4-(trifluoromethyl)coumarin (7-HFC) standard curve. One mole of 7-HFC corresponds to the metabolism of one mole of CYP2C9 substrate. **(b)** Reaction kinetics of recombinant human CYP2C9 enzyme at 37°C in the presence and absence of the indicated CYP2C9 inhibitors (the solvent control reaction contained assay buffer with 0.3% acetonitrile). **(c)** Dose-response curves for various CYP2C9 ligands of differing structural and mechanistic classes: the canonical competitive CYP2C9 inhibitor sulfaphenazole, the mechanism-based irreversible inhibitor tienilic acid, the antidepressant fluvoxamine (a competitive inhibitor of several CYP isoforms) and the CYP2C9-selective substrate diclofenac. For dose-response curves, percent activity was calculated for each concentration of inhibitor by comparison to activity of reactions containing no inhibitor. For each CYP2C9 inhibitor, IC_{50} values were derived by 4-parameter logistic curve fitting with each point representing the mean ± SEM of at least three replicates. Assays were performed according to the kit protocol.

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