ab284519 – Cytochrome P450 2C9 (CYP2C9) Inhibitor Screening Kit (Fluorometric)

For the:

- 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.

For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit:
http://www.abcam.com/ab284519

Storage and Stability

On receipt entire assay kit should be stored at -20°C, protected from light. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Materials Supplied

Item	Quantity	Storage Condition
10X CYP2C9 Assay Buffer	100 mL	-20°C
7-HFC Standard	1 vial	-20°C
CYP2C9 Inhibitor (Sulfaphenazole)	1 vial	-20°C
NADPH Generating System (100X)	1 vial	-20°C
β-NADP+ Stock (100X)	1 vial	-20°C
CYP2C9 Substrate	1 vial	-20°C
Recombinant Human CYP2C9	2 vial	-20°C

Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully utilize this assay:

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

Reagent Preparation

- Before using the kit, spin the tubes prior to opening

10X CYP2C9 Assay Buffer: Warm to room temperature (RT) before use.

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.

Assay Protocol

Standard Curve Preparation:

1. 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.

2. 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.

Test Compound preparation:

1. 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 IC50 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.

2. 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.

Δ 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 table below.*

Reaction Preparation:

1. 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
2X CYP2C9 Stock	50 µL	50 µL	50 µL	50 µL
5X Test Compound Solution	-	20 µL	-	-
5X Sulfaphenazole 300 µM Solution	-	-	-	20 µL
10X CYP2C9 Assay Buffer (+5X Solvent)	20 µL	-	50 µL	-

2. 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.

3. 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.

Δ 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 Km 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.*

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.

Δ 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.*

Calculation:

1. For each reaction well (including background and no inhibitor controls), choose two time points (T1 and T2) in the linear phase of the reaction progress curve, obtain the corresponding fluorescence values at those points (RFU1 and RFU2) and determine ΔF = (RFU2 – RFU1) and ΔT (T2 – T1). 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 (RSC) and test compound (RTC) wells to determine background-corrected reaction rates (denoted by R) for each well:

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

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

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

Δ Note: *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.*

Technical Support

For all technical or commercial enquiries please go to:

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