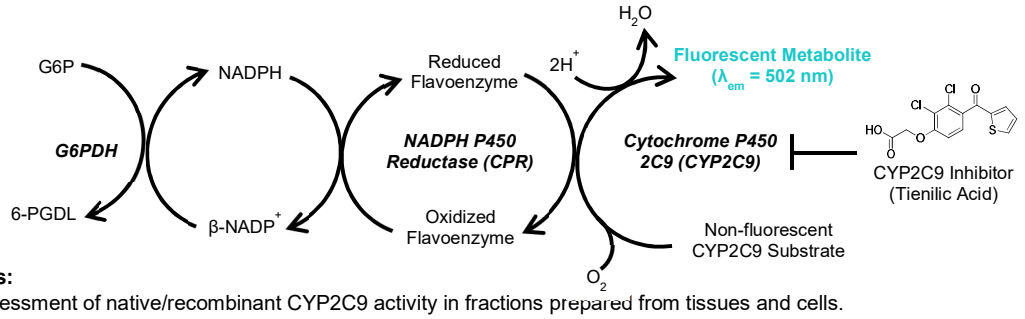
Cytochrome P450 2C9 (CYP2C9) Activity Assay Kit (Fluorometric) (#BN01069)

(Catalog # BN01069; 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. CYP2C9 is primarily expressed in the 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 Activity Assay Kit enables rapid measurement of native or recombinant CYP2C9 activity in biological samples such as liver microsomes. 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. A highly selective irreversible CYP2C9 inhibitor is provided for determination of CYP2C9 activity in heterogeneous biological samples, where other CYP isozymes may contribute to substrate metabolism. The inhibitor displays greater than 100-fold selectivity for CYP2C9 over other CYPs, ensuring targeted inhibition. CYP2C9 specific activity is calculated by running parallel reactions in the presence and absence of the selective inhibitor and subtracting any residual activity detected with the inhibitor present. The kit contains a complete set of reagents sufficient for performing 50 sets of paired reactions (in the presence and absence of inhibitor).



II. Applications:

- Rapid assessment of native/recombinant CYP2C9 activity in fractions prepared from tissues and cells.
- Screening of drugs and novel ligands for interaction with native/recombinant CYP2C9.

III. Sample Type:

- Human liver microsomes and liver S9 fractions
- Lysates of tissues and cultured cells, primary hepatocytes
- Heterologously expressed recombinant CYP2C9 preparations

IV. Kit Contents:

Components	BN01069	Cap Code	Part Number
CYP2C9 Assay Buffer	100 ml	NM	BN01069-1
7-HFC Standard	1 vial	Red	BN01069-2
CYP2C9 Inhibitor (Tienilic Acid)	1 vial	Amber	BN01069-3
NADPH Generating System (100X)	1 vial	Green	BN01069-4
β-NADP+ Stock (100X)	1 vial	Blue	BN01069-5
CYP2C9 Substrate	1 vial	Yellow	BN01069-6
Recombinant Human CYP2C9	1 vial	Violet	BN01069-7

V. User Supplied Reagents and Equipment:

- Multiwell fluorescence microplate reader
- Precision multi-channel pipette and reagent reservoir
- Anhydrous (reagent grade) acetonitrile and DMSO
- 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 CYP2C9 Assay Buffer to warm to room temperature 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. Store at -20°C, stable for at least 3 freeze/thaw cycles.
- **CYP2C9 Inhibitor (Tienilic Acid):** Reconstitute in 55 µl of acetonitrile and vortex until fully dissolved to yield a 20 mM stock solution. To obtain a 300 µM working solution of tienilic acid (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 is stable for 3 freeze/thaw cycles. The stock solution is stable for 2 months at -20°C.
- **NADPH Generating System (100X):** Reconstitute with 220 µl CYP2C9 Assay Buffer, aliquot and store at -20°C. Avoid repeated freeze/thaw cycles and keep on ice while in use.

- **β-NADP$^+$ Stock (100X):** Dissolve in 110 µl CYP2C9 Assay Buffer and vortex thoroughly to yield a 100X stock solution of NADP$^+$. Store at -20°C, stable for at least 3 freeze/thaw cycles.
- **CYP2C9 Substrate:** Reconstitute with 55 µl anhydrous reagent-grade acetonitrile and vortex until fully dissolved to obtain a 10 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 CYP2C9:** Do not reconstitute until ready to use. Reconstitute with 230 µl CYP2C9 Assay Buffer and add 20 µl of NADPH Generating System (100X). Mix thoroughly to ensure a homogenous solution (the solution will have a slightly opaque, milky appearance), aliquot and store at -80°C. Avoid repeated freeze/thaw cycles and use aliquots within one month (the Recombinant Human CYP2C9 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 2C9 (CYP2C9) Activity Assay 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 50 µl of the 200 µM solution with 950 µl CYP2C9 Assay Buffer to generate the final 10 pmole/µl (10 µM) 7-HFC Standard. Add 0, 2, 4, 6, 8, 12, 16 and 20 µl of the 10 pmole/µl 7-HFC standard into a series of wells in an opaque 96-well plate, yielding 0, 20, 40, 60, 80, 120, 160 and 200 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. 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 (Cat. #BN00514). 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 CYP2C9 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, CYP2C9 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 CYP2C9 Assay Buffer.

Notes:

- To quantify CYP2C9 specific activity in terms of sample protein content, use the Bradford reagent (Cat. #BN01026) or an equivalent protein assay.
- When measuring CYP2C9 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 CYP2C9 activity. Importantly, DMSO causes significant inhibition of CYP2C9 at final concentrations ≥0.25% (v/v). Our assay is designed to use acetonitrile at a final concentration of ≤1% (v/v), which has been shown to have little impact on CYP2C9 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 CYP2C9 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 starting with 12.5-25 µg of microsomal protein per well. For liver S9 fractions or other cellular lysates, the amount of protein required will be significantly higher. In this case, we recommend starting at 50-100 µg/well.
Note: Due to the large individual variation in CYP2C9 expression level and function, sample protein levels may need to be adjusted.

b. In addition to the test samples, prepare background control and inhibitor control (60 µM tienilic acid) wells. If desired, you may also prepare CYP2C9 enzyme positive control (PC) and PC + inhibitor wells using the Recombinant Human CYP2C9 and tienilic acid 300 µM solution (for a 60 µM final concentration of tienilic acid). Adjust the volume of test sample, inhibitor control and positive control wells to 70 µl/well with CYP2C9 Assay Buffer. For measurement of CYP2C9 activity in the presence of test ligands, replace CYP2C9 Assay Buffer with 5X concentrated test ligand solution:

	Test Sample	+ Inhibitor Control	Background	2C9 PC	PC + Inhibitor
P450 Reaction Mix (2X)	50 µl	50 µl	—	—	—
Recombinant Human CYP2C9	—	—	—	25 µl	25 µl
Tienilic Acid 300 µM Solution (5X)	—	20 µl	—	—	20 µl
CYP2C9 Assay Buffer	20 µl	—	50 µl	45 µl	25 µl
Test Ligand (5X)	—	—	20 µl	—	—

c. Incubate the plate for at least 30 min at 37°C to allow the inhibitor tienilic acid or any test ligands to interact with CYP2C9 (tienilic acid exhibits time-dependent inactivation of CYP2C9, necessitating a 30 min pre-incubation period to ensure complete inhibition). The pre-incubation time can be optimized for each test ligand depending on mechanism of action.

d. 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 reactions, but can be scaled depending upon the number of reactions to be performed. Start the reaction by adding 30 µl of the CYP2C9 Substrate/NADP$^+$ (3X) mixture to each well using a multichannel pipette, yielding a final reaction volume of 100 µl/well.

Notes:

- The Recombinant Human CYP2C9 preparation may settle and should be thoroughly mixed before dispensing.
- The CYP2C9 Substrate is also metabolized by CYP isoforms 2B6 and 2C19, necessitating the use of the selective inhibitor tienilic acid to determine the contribution of CYP2C9 in heterogeneous biological samples. The concentration of tienilic acid used in our assay is >25-fold greater than the K_i for recombinant CYP2C9. In human liver microsomes, this concentration typically results in 35-40% inhibition of 7-HFC formation, which represents the CYP2C9-mediated metabolic activity. The contribution of the off-target CYP isoforms (2B6 and 2C19) to substrate metabolism may be tested using the inhibitor ticlopidine (at a final concentration of 30 µM, which produces complete inhibition of both CYP2B6 and CYP2C19.

4. **Measurement:** Immediately (within 1 min) measure the fluorescence at Ex/Em = 415/502 nm in kinetic mode for 60 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 the content of active CYP2C9 in the sample.

   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 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 60 µM tienilic acid positive inhibition control (I) to determine the background-corrected change in fluorescence intensity for each well.

   Note: In our experience, the CYP2C9 Substrate does not undergo appreciable non-enzymatic conversion to the fluorescent product. Thus, the background control (BC) well rate calculation may yield a negative value, in which case, the BC value may be ignored. Calculate the specific fluorescence generated by CYP2C9 activity (denoted by C) by subtracting the positive inhibition control from each sample:

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

CYP2C9 metabolic activity is obtained by applying the C_S values to the 7-HFC standard curve to get B pmole of substrate metabolized to 7-HFC by CYP2C9 during the reaction time.

$$\textbf{Cytochrome P450 2C9 Specific Activity} = \frac{B}{\Delta T \times P} = \textbf{pmole/min/mg} = \textbf{µU/mg}$$

Where: $\mathbf{B}$ is the amount of 7-HFC produced, calculated from the standard curve (in pmole)
$\Delta\mathbf{T}$ is the linear phase reaction time $T_2 - T_1$ (in minutes)
$\mathbf{P}$ is the amount of protein in the well (in mg)

CYP2C9 Unit Definition: One unit of CYP2C9 activity is the amount of enzyme that generates 1 µmole of 7-HFC per min by hydrolysis of 1 µmole fluorogenic substrate at 37°C and pH 8.

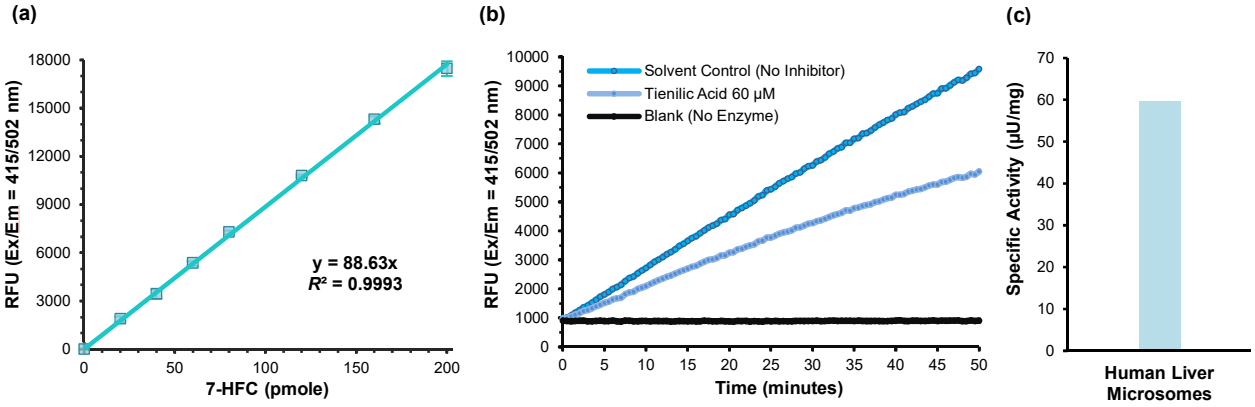


Figure: (a) 7-hydroxy-4-(trifluoromethyl) coumarin (7-HFC) standard curve. One mole of 7-HFC corresponds to the metabolism of one mole of CYP2C9 substrate. **(b)** Reaction kinetics of fluorogenic substrate metabolism in donor-pooled human liver microsomes (0.125 mg/mL) at 37°C in the presence and absence of the CYP2C9 inhibitor tienilic acid (the solvent control contained assay buffer with 0.3% acetonitrile). **(c)** Specific activity of CYP2C9 in human liver microsome sample. Assays were performed according to the kit protocol.

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