RECO® System
Recombinant Human CYP2C9 Simplified Reconstitution Assay
Product # P2362

1.0 INTRODUCTION

PanVera's RECO® Systems greatly simplify the steps involved in reconstituting purified cytochrome P450 isozymes and their associated proteins. The amount of reconstitution mix and the volume used for an assay will depend on several factors:

- sensitivity of the analytical assay for the metabolites and catalysis products
- kinetic parameters of enzyme catalysis for a particular substrate and P450, if known
- incubation period of the assay
- solubility of the test compound or substrate
- preference of individual research groups

Details of general assays[1,2,3] and reconstitution methods[4,5,6] have been published. Most details of specific substrate assays for individual isozymes of cytochrome P450 have also already been published:

Isozyme	Substrate
CYP1A1	Ethoxyresorufin[7]
CYP1A2	Methoxyresorufin[7]
CYP3A4	Nifedipine, Testosterone, Midazolam, Erythromycin[4,5,8,9]
CYP2C9	Diclofenac[10,11]
CYP2C19	Mephenytoin[11,12,13]
CYP2D6	Bufuralol, Debrisoquine, Dextromethorphan[11,14]
CYP2E1	N-nitrosodimethylamine, Chlorzoxazone[15,16]

Individual laboratories have modified the assays to suit their personal preferences. When using a substrate whose kinetic parameters are not known, the following conditions may serve as a useful starting point:

Reaction Volume	0.5-1.0 mL
CYP2C9 Enzyme Mix	50-100 pmol
Substrate Concentration	100-500 μM
Incubation Time	5-10 min

A pre-incubation period of 2-3 minutes is often performed before the reactions are initiated by the addition of the final compound (substrate, or either NADPH or a regenerating system, if preferred). The linearity of the assay and kinetic parameters should be determined by standard methods. Reactions are stopped by adding either methanol, acetonitrile, or acid (final concentration of ≥20%) to the incubation mixes and then placing them on ice. If an internal standard is to be used for the analytical assay, it can often be included in the stop mixture. In some cases, extracting the metabolites from the reaction mix is useful, as it enhances detection by concentrating the metabolites and removing water-soluble compounds that interfere with the analysis procedure. Typically, 1.5-5 volumes of methylene chloride or ethyl acetate can be used to extract the metabolites, although other salt additives that affect the pH and/or enhance the extraction procedure may be necessary. In cases where extraction can be avoided, the reaction is stopped (as above) and the mixture is centrifuged for 10 minutes at 4°C in order to pellet the protein. The supernatant can then be used directly for analysis.

2.0 DESCRIPTION

2.1 Materials Supplied

CYP2C9 RECO® System Enzyme and Buffer Mixes

- Enzyme Mix: Part #P2387: 0.5 μM CYP2C9, 1.0 μM NADPH P450 reductase, 0.5 μM cytochrome b_5, 0.5 μg/μL CHAPS, 0.1 μg/μL liposomes [dilauroyl phosphotidylcholine, dioleoyl phosphotidylcholine, dilauroyl phosphotidylserine (1:1:1)], 3 mM reduced glutathione, and 50 mM HEPES/KOH (pH 7.4).
- 5 X Buffer Mix: Part #P2388**:** 0.5 M Tris-HCl, pH 7.5.

2.2 Materials Required but Not Supplied

- Stock solution 100 mM NADPH or a regenerating system (made fresh in water just before starting the reactions)
- Stock solution of test drug/substrate, Example: 5 mM diclofenac
- Distilled deionized water

2.3 Safety Precautions

Normal precautions exercised in handling laboratory reagents should be followed. The reagents supplied are not considered hazardous according to 29 CFR 1910.1200. The chemical, physical, and toxicological properties of these products may not, as yet, have been thoroughly investigated. We recommend using gloves, lab coats and eye protection when working with any chemical reagents.

3.0 PROCEDURE

This assay is specific for the measurement of CYP2C9, and to some extent CYP2C19. The assay can be done in a single microcentrifuge tube and no extraction is required. No internal standard is used, so a standard curve should be performed for each set of assays. The final concentration of P450 should be 25 pmol/mL and diclofenac should be 100μM. For the RECO® System, use either 2.5 pmol of CYP2C9 in a reaction volume of 100 μL or 6.25 pmol of CYP2C9 in a reaction volume of 250 μL.

For a 100 μL reaction:

1. Prepare NADPH and test drug/substrate stock solutions.
2. Thaw enzyme and buffer mixes rapidly and place on ice.
3. Working on ice, combine the following to make the buffer/substrate solution:

Enzyme mix	6.25 μL
CYP2C9 Buffer Mix	20 μL
Water	67.75 μL
Substrate (5 mM diclofenac)	2 μL
Total volume	96 μL

4. Pre-incubate the buffer/substrate solution at 37°C for 3 minutes.
5. Start the reaction by adding 4 μL of 50 mM NADPH.
6. Incubate at 37°C for the desired reaction time.
7. Stop the reaction by adding 20 μL of 94:6 (v/v) acetonitrile:glacial acetic acid.
8. Vortex briefly and place on ice. Centrifuge the tubes for 10 minutes at 4°C to remove insoluble material. Analyze by HPLC.
9. HPLC: C18 column, 4.6 mm x 70 mm, flow rate 1.2 mL/ min, detection UV 280 nm
   Solvent A: 30% acetonitrile containing 1 mM perchloric acid
   Solvent B: methanol
   Initial conditions: 30% solvent B. After 1 minute start gradient to 100% Solvent B over 6 minutes.

6.0 REFERENCES

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