

Protocol of P450-Glo™ CYP2C9 Assay for High-throughput Screening

DOCUMENT: P450-Glo™ CYP2C9_TOX21_SLP_Version1.0

TITLE: Protocol of P450-Glo™ CYP2C9 Assay for High-throughput Screening

ASSAY REFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
CYP2C9	N/A	Human	Enzyme	Luminescence	Promega Corporation	Cell-free cytochrome P450

QUALITY CONTROL PRECAUTIONS:

1. Avoid repeated freeze-thaw cycles of substrate and enzyme.
2. Dispense the unused Luciferin-H and CYP2C9 membranes into single-use aliquots and store at -70°C.
3. Keep the mixtures on ice during assay run.

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
-P450-Glo™ CYP2C9 Screening System	-Promega	-Promega/V9790
-Sulfaphenazole (Positive control compound)	-Sigma Aldrich	-Sigma Aldrich/S0758
-1536-well medium binding white solid plates	-Greiner Bio-one	-Greiner Bio-one/789175-F
-BioRAPTR FRD	-Beckman Coulter	-Beckman Coulter
-Pintool station	-Wako	-Wako
-ViewLux plate reader	-Perkin Elmer	-Perkin Elmer

PROCEDURE:

1. Enzyme-substrate mixture:

- i. Luciferin-Free water = 21.58uL
- ii. Potassium Phosphate Buffer, 1M = 1.25uL
- iii. 5mM Luciferin-H = 1.0uL
- iv. CYP2C9 membranes = 0.5uL
- v. 0.2% BSA (7.5%BSA stock) = 0.67uL
- vi. Final Volume = 25uL

2. NADPH regeneration solution:

- i. Luciferin-Free water = 22.0uL
- ii. Solution A = 2.5uL
- iii. Solution B = 0.5uL
- iv. Final Volume = 25.0uL

3. Assay Protocol

- i. Two uL of enzyme-substrate mixture was dispensed dispenser in 1536-well medium-binding white solid plates using BioRAPTR dispenser.
- ii. The positive control and test compounds were transferred at 23nL to 1-4 and 5-48 columns of the assay plates respectively using Pintool station.

Positive control plate format: Column 1: two-fold sixteen-point titration starting at 10mM (final = 57.5uM) duplicates; Columns 2 & 3 (top halves): 1.25mM (final = 7.2uM) and 625uM (3.6uM) Sulfaphenazole respectively; Columns 2 & 3 (bottom halves) and 4: DMSO.
Control used is Sulfaphenazole [10mM made in DMSO (final = 57.5uM)]
- iii. The assay plates were incubated for 10min at room temperature.
- iv. The reaction was initiated through the addition of 2uL NADPH regeneration solution using BioRAPTR dispenser.
- v. The assay plates were incubated for 60min at 37°C to allow the reaction to continue before it was stopped with a detection reagent.
- vi. Four uL of detection reagent was added using BioRAPTR dispenser.
- vii. The assay plates were incubated for 20min at 37°C.
- viii. The luminescence intensity was quantified using ViewLux plate reader (Exposure time: 10sec).

ASSAY PERFORMANCE:

Online Validation	P450-Glo™ CYP2C9 assay
AC ₅₀ (uM)	0.19 ± 0.02 (n = 27)
CV*	4.66 ± 0.91 (n = 27)
S/B	23.8 ± 1.24
Z	0.83 ± 0.03

⊙ CV values shown represent average of DMSO column from each plate.