Protocol of P450-Glo™ CYP3A4 Assay for High-throughput Screening

DOCUMENT: P450-Glo™ CYP3A4_TOX21_SLP_Version1.0
TITLE: Protocol of P450-Glo™ CYP3A4 Assay for High-throughput Screening

ASSAY RFERENCES:

Assay Target	Cell Lines	Species	Tissue of Origin	Assay Readout	Assay Provider	Toxicity Pathway
CYP3A4	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-PPXE and CYP3A4 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™ CYP3A4 Screening System (Luciferin-PPXE) DMSO Tolerant Assay	-Promega	-Promega/V9910
-Ketoconazole (Positive control compound)	-Sigma Aldrich	-Sigma Aldrich/K1003
-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. 100mM Tris-HCl = 11.975uL
    ii. 50mM Luciferin-PPXE = 0.025uL
    iii. CYP3A4 membranes = 0.5uL
    iv. 0.2% BSA (7.5% stock) = 0.67uL
    v. Luciferin-Free water = 11.83uL
    vi. Final volume = 25.0uL

2. NADPH regeneration solution:
    i. Luciferin-Free water = 12.0uL
    ii. Potassium Phosphate Buffer, 1M = 10.0uL
    iii. Solution A = 2.5uL
    iv. Solution B = 0.5uL
    v. 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) Ketoconazole respectively; Columns 2 & 3 (bottom halves) and 4: DMSO.

        Control used is Ketoconazole [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 room temperature 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 room temperature.
    viii. The luminescence intensity was quantified using ViewLux plate reader (Exposure time: 60sec).

ASSAY PERFORMANCE:

Online Validation	P450-Glo™ CYP3A4 assay
AC_{50} (uM)	0.02 ± 0.001 (n = 26)
CV*	2.13 ± 0.36 (n = 27)
S/B	4.36 ± 0.05
Z	0.88 ± 0.05

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