

Protocol of P450-Glo™ CYP2D6 Assay for High-throughput Screening

DOCUMENT: P450-Glo™ CYP2D6_TOX21_SLP_Version1.0

TITLE: Protocol of P450-Glo™ CYP2D6 Assay for High-throughput Screening

ASSAY REFERENCES:

| Assay Target | Cell Lines | Species | Tissue of Origin | Assay Readout | Assay Provider | Toxicity Pathway |
|--------------|------------|---------|------------------|---------------|---------------------|---------------------------|
| CYP2D6 | 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-ME EGE and CYP2D6 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™ CYP2D6 Screening System | -Promega | -Promega/V9890 |
| -Quinidine (Positive control compound) | -Sigma Aldrich | -Sigma Aldrich/Q3625 |
| -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 = 18.93uL (this addition is to make up the volume)
- ii. Potassium phosphate buffer, 1M = 5.0uL
- iii. 10mM Luciferin-ME EGE = 0.15uL (10mM Luciferin-ME EGE solution was prepared by dissolving 900ug of Luciferin-ME EGE in 265ul of acetonitrile and mix vigorously)
- iv. CYP2D6 membranes = 0.25uL
- 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: Columns 1: two-fold sixteen-point titration starting at 250uM (final = 1.44uM) duplicates; Columns 2 & 3 (top halves): 125uM (final = 0.72uM) and 62.5uM (0.36uM) Quinidine respectively; Columns 2 & 3 (bottom halves) and 4: DMSO.

Control used is Quinidine [250uM made in DMSO (final = 1.44uM)]

- 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: 10sec).

ASSAY

| Online Validation | P450-Glo™ CYP2D6 assay |
|-----------------------|-------------------------|
| AC ₅₀ (nM) | 5.38 ± 0.26 (n = 27) |
| CV* | 2.22 ± 0.77 (n = 27) |
| S/B | 18.70 ± 0.37 |
| Z | 0.92 ± 0.03 |

PERFORMANCE:

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