

High Throughput Bioluminescent Assays for P450s and MAO

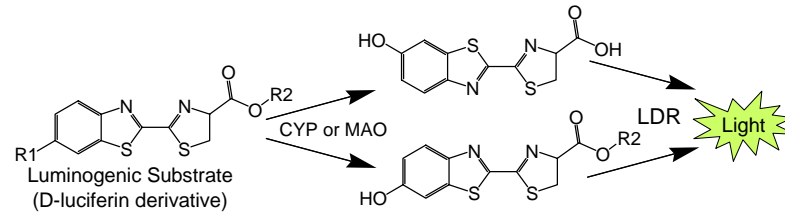
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Abstract

Bioluminescence offers significant advantages for configuring sensitive, simple to perform, homogenous high through-put assays. We have developed the bioluminescent P450-Glo™, Cytochrome P450 assays and MAO-Glo™, Monoamine Oxidase Assay for predicting the metabolism-based drug-drug interaction potentials of new chemical entities. P450-Glo™ and MAO-Glo™ Assays rely on luminogenic probe substrates and the light generating reaction of firefly luciferase to measure the impact of test compounds on several P450s and MAO-A and MAO-B. P450-Glo™ assays are also used in a cell-based method for measuring P450 gene induction at the P450 enzyme level. This suite of assays is ideal for profiling compounds against multiple metabolic enzymes with a common luminescent readout.

Assay Scheme and Basic Protocol

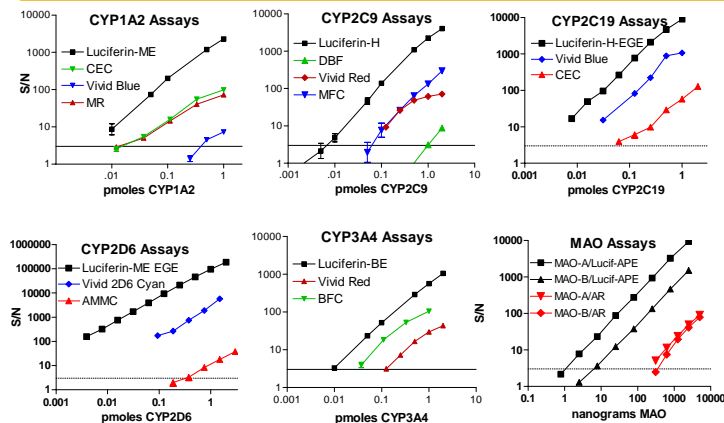


1. Enzyme/substrate selectivity depends on the nature of R1 and R2
2. Incubate cytochrome P450 (CYP) or monoamine oxidase (MAO) enzyme with appropriate luminogenic substrate under reactive conditions in a multi-well plate format.
3. Terminate enzyme reactions and initiate luminescence by adding one volume luciferin detection reagent (LDR).
4. Read luminescence with a plate-reading luminometer.

Enzyme/Substrate Selectivity

Substrate	R1	R2	Enzyme	Assays
Luciferin-ME		-H	CYP1A2, 2C8, 2C9, 2J2, 4A11, 4F3B, 19	Enzymes & Cells
Luciferin-CEE		-H	CYP1A1, 1B1, 3A7	Enzymes & Cells
Luciferin-H	H-	-H	CYP2C9	Enzymes & Cells
Luciferin-BE		-H	CYP3A, 4F12	Enzymes
Luciferin-PFBE		-H	CYP3A	Enzymes & Cells
Luciferin-PPXE prototype		-H	CYP3A	Enzymes & Cells (DMSO insensitive)
Luciferin-ME-EGE			CYP1A, 2D6	Enzymes
Luciferin-H-EGE	H-		CYP1A, 2C19	Enzymes
Luciferin-APE			MAO-A, MAO-B	Enzymes

Assay Sensitivity: Luminescence > Fluorescence



Recombinant human enzymes assayed at substrate K_m , S/N=3=LOD. MR=methylresorufin, BFC=benzylfluorocoumarin, DBF=dibenzylfluorescein, CEC=cyanoeethylcoumarin, AMMC=(N,N-diethyl-N-methylammonium)ethyl-m-methylcoumarin, AR=Amplex Red

Inhibition: Glo™ Assay IC₅₀s/Literature IC₅₀s

CYP1A1/luciferin-CEE
Ketoconazole, **25.0/7.4**
 α -NA, **0.4/0.4**
Phenacetin, **22.6**
Quinidine, **3.2/1.4**
Retinoic Acid, **18.0**

CYP1A2/luciferin-ME
Fluvoxamine, **0.12/0.04**
Furafylline, **0.4/0.7**
 α -NA, **0.1/0.08**
 β -NA, **0.6**

MAO-A/luciferin-APE
Clorgyline, **0.003/0.004**
Deprenyl, **4/5**
Phenylethylamine, **45/80**
Serotonin, **45/80**

CYP2C9/luciferin-H
Diclofenac, **2.4/2.1-2.8**
Fluvoxamine, **8.7/2.2**
Ibuprofen, **64.2/145**
Sulfaphenazole, **0.2/0.2**
(S)-(-)-Warfarin, **4.6/14**
Troglitazone **4.4/3**

CYP2C19/luciferin-EGE
Fluvoxamine, **0.3/0.1**
Furafylline, **8.0/2.8-9.0**
Quinidine, **0.01/0.01**
Quinine, **11.0/20**
Verapamil, **67.0/60**

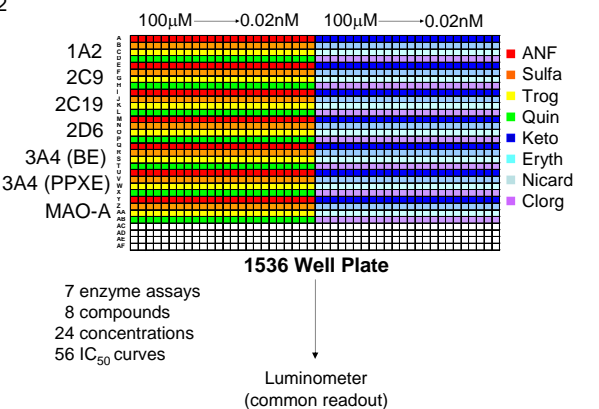
IC₅₀s (μ M) measured against recombinant enzymes with Glo™ Assays (bold red font) are compared to published values (black font) that used various conventional assays. Literature references are listed in Cali, JJ *et al.* (2006) *Exp. Op. Drug Metab. Toxicol.* 2(4), 629-645 and Valley, M. *et al.* (2006) *Anal. Bioch.* 359(2):238-46. Numbers in italics are K_i values. "+" indicates positive cooperativity.

CYP2D6/luciferin-ME EGE
Bupropion, **22.8/45**
Bupropion, **33.9**
Clotrimazole, **22.6**
Debrisoquine, **77.4/46**
Dextromethorphan, **6.8/7**
Fluoxetine, **6.0/2.0**
Fluvoxamine, **3.4/4.9**
Haloperidol, **1.2/3**
Nicardipine, **8.0/2.8-9.0**
Quinidine, **0.01/0.01**
Quinine, **11.0/20**
Verapamil, **67.0/60**

CYP3A4/luciferin-BE
Azamulin, **0.1/0.03-0.12**
Clotrimazole, **0.006/0.002-0.02**
Disopyramide, **29.0/~30**
Erythromycin, **1.2/1.8-74**
Fluvoxamine, **15.2/10.7**
Haloperidol, **+/+**
Ketoconazole, **0.1/0.1**
Midazolam, **17.4/1.3-59.8**
 α -NA, **+/+**
Nifedipine, **+/+**
Omeprazole, **61.0/78**
Testosterone, **+/+**
Troleandomycin, **0.2/0.3-6.1**
Verapamil, **0.4/0.4-8.4**

Compound Profiling

Assaying Multiple Enzymes Against Multiple Compounds With a Common Luminescent Readout



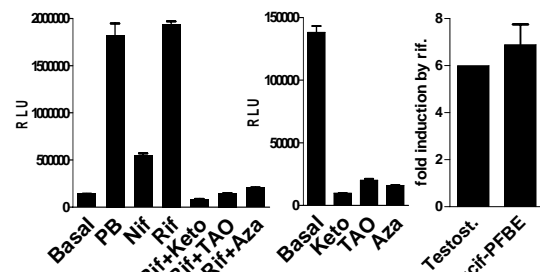
Cell-Based P450-Glo™ Assays

Protocol

- Treat monolayer cultures (e.g. hepatocytes) with test compounds (e.g. P450 gene inducer).
- Add luminogenic P450 substrate to cell culture medium, incubate 3-4 hours.
- Remove sample of medium and combine with P450-Glo™ luciferin detection reagent (culture remains intact).
- Read luminescence.
- Measure viable cell count per well (e.g. CellTiter-Glo™). Normalize P450-Glo™ values to cell number.
- Alternate homogenous method: add LDR direct to well and read.

CYP3A Induction in Primary Hepatocytes

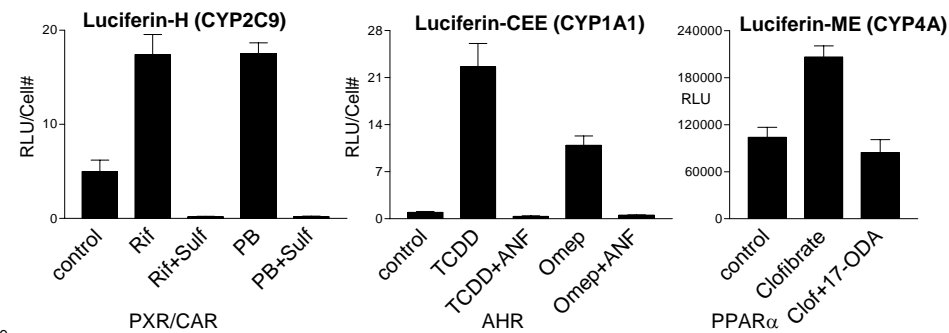
CYP3A/Luciferin-PFBE Assay



Fresh human hepatocytes in 96-well plate
PB=0.5mM phenobarbital, Rif=10 μ M rifampicin, Nif=50mM nifedipine, keto=10 μ M ketoconazole, TAO=0.1mM troleandomycin, Aza=10 μ M azamulin

Cryopreserved human hepatocytes. testosterone hydroxylation assay correlates well with Luciferin-PFBE Assay

CYP2C9, CYP1A and CYP4A Induction in Human Hepatocytes



Sulf=Sulfaphenazole, TCDD=tetrachlorodibenzyl dioxin, omeprazole, ANF=a-naphthoflavone, clof=clofibrate, 17-octadecynoic acid

Summary

Here we demonstrated the utility of bioluminescent P450-Glo™ and MAO-Glo™ assays for measuring inhibition and induction of metabolic enzymes by xenobiotics. The need to detect such effects is underscored by the prevalence of adverse drug-drug interactions that occur as a consequence of CYP450 and MAO enzyme inhibition and P450 gene induction. The Glo™ enzyme assays eliminate the need for time consuming LC/MS approaches and fluorescence interference common to fluorescent probe based assays. Because each Glo™ Assay uses a common luminescent readout, multiple enzymes can be screened against a common set of test compounds in a single configuration. The IC₅₀s we measured with Glo™ Assays correlate well with literature values measured by different assay technologies. When used for a cell based assay format P450-Glo™ was simple to perform in multi-well plates and predicted detected P450 gene inductions at the level of enzyme activity.