Miniaturization and automation of cytochrome P450 inhibition assays

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Abstract

Members of the cytochrome P450 (CYP) family of metabolic enzymes are recognized as a major factor in adverse drug interactions, and screening new chemical entities (NCEs) for P450 inhibition potential is a critical step in the drug discovery process. Hence, there is a need for rapid, reliable assessment of the P450 inhibition potential of NCEs. To this end, cytochrome P450 inhibition assays were developed to detect drug-drug interactions (DDI) using microsomes containing cDNA-expressed human CYP1A2, 2C9, 2C19, 2D6, or 3A4 with cytochrome P450 reductase (P450 Baculosomes® Reagents) and fluorogenic Vivid® substrates in a 384-well plate format using a reaction volume of 15 µL. Several factors were determined to be especially advantageous in developing a streamlined assay protocol compatible with automation. The assay reagents were shown to be stable at room temperature, enabling automated reagent dispensing over the course of several hours. The rate of metabolite formation in each assay was observed to be linear for at least 30 minutes at room temperature, allowing use of a uniform reaction time. Each assay was compatible with the presence of 1% DMSO, allowing standardized compound handling. Use of a universal stop solution, which lacks a volatile organic solvent, enabled the assays to be performed in endpoint mode. After stopping the reaction, the fluorescent assay signal was found to be stable for several hours. Multiple structurally unique substrates were used for CYP3A4 and 2C9 to detect potential probe substrate-dependent inhibition, heteroactivation, or other complex DDI. The use of multiple substrates with distinct optical properties, as well as rigorous intra-plate controls, were also used to identify and account for possible test compound autofluorescence. The miniaturized P450 inhibition assays are robust, with average Z′-factor values >0.8 and inhibition results in agreement with those of known inhibitors for each P450. The assays described here are offered and used in a custom P450 profiling service.

Methods

Materials – P450 Baculosomes® (microsomes prepared from insect cells containing a single human P450 and P450 reductase), control Baculosomes® (microsomes prepared from insect cells infected with a control virus), Regeneration System (glucose-6-phosphate dehydrogenase and glucose-6-phosphate), $NADP^+$, Vivid® Substrates, and assay buffer were from Invitrogen Corp. (Madison, WI). Assay plates were 384-well low volume black polystyrene plates (cat. no. 3677) from Corning (Acton, MA).

Automation Equipment – A Biomek FX Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA) was used for test compound/DMSO control stamping and to dispense all assay reagents. In order to increase throughput, a Cytomat Microplate Hotel (Beckman-Coulter, part no. 394597) was partnered with the Biomek FX for extra storage of assay plates, reagents, and tip boxes.

Compound Preparation – Serial dilutions of test compounds were performed in 100% DMSO at 100X the final assay concentration. Compounds were then diluted to 3X concentration using assay buffer. Assay plates were then stamped with 5 µL of 3X test compound to sample wells or 5 µL of 3% DMSO to control wells.

Vivid P450 Inhibition Assay – All steps were conducted at room temperature. 5 µL of 3X P450 Baculosomes®/Vivid® Substrate or 3X control Baculosomes®/Vivid® Substrate in assay buffer (0.2X assay buffer for CYP2C9 assays) were dispensed to the assay plate and shaken on an orbital shaker for 30 seconds. Each test compound concentration was tested in duplicate in the presence of P450 Baculosomes® to assess inhibition as well as in the presence of control Baculosomes® to detect autofluorescent compounds. 1% DMSO in the control wells was used in the presence of P450 Baculosomes® (n=16) to represent 100% activity ("signal") or in the presence of control Baculosomes® (n=16) to represent 0% activity ("background"). Plates were incubated for 10 min prior to starting the reactions with 5 µL of 3X Regeneration System/$NADP^+$ to all wells. Plates were shaken for 30 sec as above and incubated for 25 min. The reaction was stopped by addition of 5 µL of 0.5 M tris base (pH 10.5) to all wells and shaken for 30 sec as above. Plates were then read on a Safire (Tecan, Durham, NC) plate reader using the fluorescent excitation and emission settings indicated in Table 1.

Data Analysis

Percent Activity

$$\% \text{ activity} = [(RFU_{sample} - RFU_{background})/(RFU_{signal} - RFU_{background})] \times 100\%$$

where RFU = "relative fluorescence units", fluorescent intensity

Z′-Factor

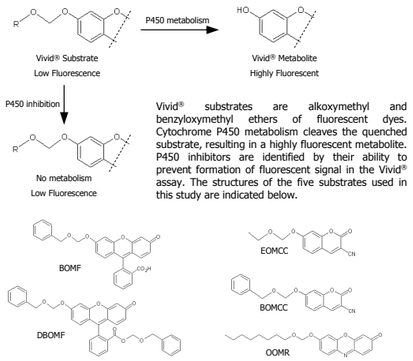
$$Z' = 1 - [(3xStd\ dev_{signal\ RFU} + 3xStd\ dev_{background\ RFU})/(mean_{signal\ RFU} - mean_{background\ RFU})]$$

Z′-factor is a parameter used to measure the robustness of an assay, taking into account both assay window and variability. Values >0.5 are generally considered suitable for high-throughput screening, while a value of 1 indicates a theoretical ideal assay with no variability (Zhang *et al*, 1999).

IC_{50} Determination

Percent activity of sample wells was plotted versus the log value of each test compound concentration to generate an 11-point dose-response curve. Nonlinear regression was performed with GraphPad Prism software (San Diego, CA) using an equation for sigmoidal dose-response (variable Hill slope) to determine the IC_{50} value.

Figure 1 – Vivid® Assay Principle



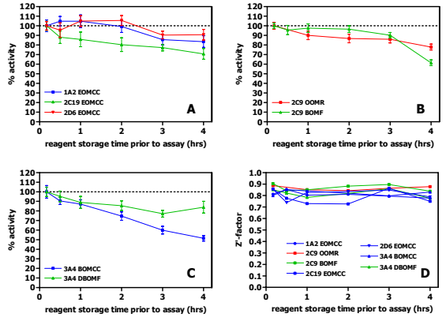
Vivid® substrates are alkoxymethyl and benzyloxymethyl ethers of fluorescent dyes. Cytochrome P450 metabolism cleaves the quenched substrate, resulting in a highly fluorescent metabolite. P450 inhibitors are identified by their ability to prevent formation of fluorescent signal in the Vivid® assay. The structures of the five substrates used in this study are indicated below.

Table 1 – Vivid® Assay Conditions

Assay	[P450] (nM)	[Substrate] (µM)	Excitation/Emission (nm)
1A2 EOMCC	5	3	415/460
2C9 BOMF	20	1	490/520
2C9 OOMR	20	1	550/590
2C19 EOMCC	5	10	415/460
2D6 EOMCC	20	10	415/460
3A4 BOMCC	2.5	10	415/460
3A4 DBOMF	2.5	2	490/520

Each assay also contained 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 30 µM $NADP^+$ to supply a constant amount of the cofactor NADPH. The buffer was 100 mM potassium phosphate pH 7.4, except for the CYP2C9 assays, which contained 60 mM potassium phosphate pH 7.4. All assays also contained 1% DMSO, introduced as the test compound vehicle solvent or included in control assay wells. A 12 nm bandwidth was used for both excitation and detection of light emission, centered on the wavelengths listed above.

Figure 2 – Room Temp Reagent Stability



Each 3X Baculosomes®/Vivid® Substrate reagent was prepared in assay buffer and incubated at room temperature for varying time periods prior to measurement of activity (Figures 2A, 2B, 2C). Note that while not all P450/substrate reagents retain full activity at later timepoints, the quality of the data remains robust even when stored for 4 hours as indicated by Z′-factor values >0.7 (Figure 2D). The 3X Regeneration System/$NADP^+$ reagent was found to be stable for at least 4 hours when stored at room temperature (data not shown).

Figure 3 – Assay Linearity

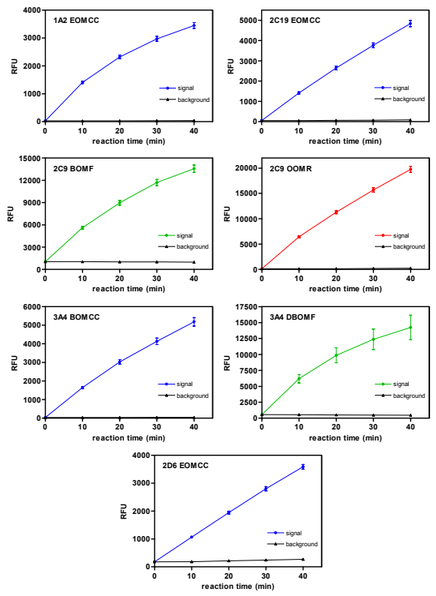
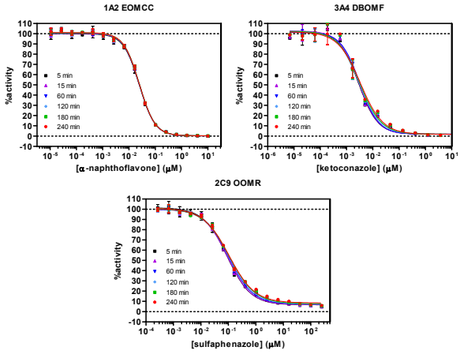


Figure 3 depicts the generation of fluorescent metabolite in each assay over time at room temperature. "Signal" indicates the fluorescent intensity measured in the presence of P450 Baculosomes®, while "background" represents the fluorescent intensity of wells containing control Baculosomes®. Error bars represent the standard deviation of 3 replicate assay wells. A 25-minute incubation time was used for screening test compounds in the Vivid® P450 inhibition assays.

Figure 4 –Signal Stability After Addition of Stop Solution



Because the assays were performed using a single plate read in endpoint mode, a basic tris solution (pH 10.5) was used to stop the reaction at the end of the desired incubation time. After addition, the pH value was increased from 7.4 to approximately 9.5. Acetonitrile, a volatile solvent commonly used as a stop solution component in other methods, was not needed for the Vivid® assays. The pH increase was sufficient to stop P450 activity, and following addition, the signal was found to be stable for several hours in all assays. Example data illustrating the stability of IC_{50} measurements is shown for assays utilizing cyanocoumarin (EOMCC), fluorescein (DBOMF), and resorufin-based (OOMR) substrates.

Table 2 – Vivid® Assay Example IC_{50} Values (µM)

Compound	1A2 EOMCC	2C9 BOMF	2C9 OOMR	2C19 EOMCC	2D6 EOMCC	3A4 BOMCC	3A4 DBOMF
amitriptyline	>100	>100	60	9.9	2.5	23	32
astemizole	>100	13	7.7	7.5	5.8	1.2	2.5
bufuralol	>100	>100	>100	>100	3.8	>100	>100
bupropion	ND	>100	>100	11	2.0	>100	>100
clotrimazole	10	0.44	0.20	0.97	9.1	0.013	0.011
dextromethorphan	ND	450	>1000	180	0.66	120	54
diclofenac	>1000	4.9	3.0	190	630	>1000	750
fluoxetine	41	23	12	0.46	0.92	11	21
furafylline	2.6	>300	>300	120	160	110	60
imipramine	>100	>100	98	23	0.69	21	49
ketoconazole	65	7.6	3.3	5.1	7.2	0.13	0.016
mifepristone	>100	3.4	3.1	6.5	11	1.2	2.7
miconazole	2.8	1.1	2.2	0.069	0.43	0.64	0.44
nifedipine	7.9	1.7	1.0	1.3	>100	0.72	0.29
α-naphthoflavone	0.029	4.2	2.4	6.8	ND	24	9.6
omeprazole	250	5.9	2.9	1.7	120	24	31
phenacetin	9.1	560	590	390	ND	470	150
propranolol	1.1	>100	>100	>100	1.1	>100	>100
quinidine	ND	>100	>100	>100	0.008	20	29
saquinavir	ND	9.2	5.8	15	6.2	2.3	3.2
sulfaphenazole	ND	0.31	0.13	>300	ND	190	>300
terfenadine	78	15	12	10	0.55	0.34	0.86
testosterone	ND	88	45	69	>100	*A*	45
troglitazone	36	1.0	0.64	3.1	32	2.1	3.9
troleandomycin	ND	ND	ND	ND	>100	1.6	5.0
verapamil	ND	>100	51	>100	88	3.2	9.2
warfarin	ND	11	9.0	>200	>200	ND	>200

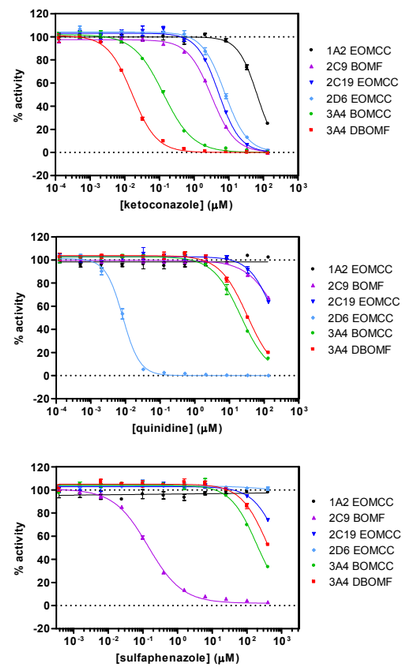
ND indicates inhibition not detected, *A* indicates apparent activation of P450-mediated probe substrate metabolism by test compound, "**>**" is used to denote cases where inhibition was observed but ≥50% inhibition was not achieved at the highest concentration of test compound used. None of the compounds above exhibited fluorescent interference in any of the Vivid® assays.

Table 3 – Vivid® Assay Robustness

Assay	Signal-to-background ratio (avg ± std dev)	% std dev (signal, background)	Z′-Factor (avg ± std dev)
1A2 EOMCC	63±4	6.2, 2.8	0.81±0.03
2C9 BOMF	10.8±1.1	3.0, 1.7	0.90±0.02
2C9 OOMR	56±14	2.8, 6.4	0.91±0.02
2C19 EOMCC	43±3	4.7, 3.0	0.85±0.02
2D6 EOMCC	10.2±0.7	4.7, 2.4	0.84±0.04
3A4 BOMCC	160±20	4.7, 4.8	0.86±0.04
3A4 DBOMF	33±9	3.0, 3.3	0.90±0.03

Table 3 lists performance parameters for each assay, calculated using the control wells on a single assay plate taken from five independent experiments. Signal ("100% activity") was calculated from the fluorescence intensity of wells containing P450 Baculosomes® (n=16), and background ("0% activity) was determined from the fluorescence intensity of wells containing control Baculosomes® (n=16), both in the presence of 1% DMSO. All of the assays were very robust, with Z′-factor values ≥0.81 and low % standard deviation values.

Figure 5 – Example Inhibition Curves



Test compounds were tested against the panel of P450 inhibition assays using an 11-point titration (n=2) and 3-fold serial dilution scheme. The example data above illustrates screening results using the potent CYP3A4 inhibitor ketoconazole, selective CYP2D6 inhibitor quinidine, and selective CYP2C9 inhibitor sulfaphenazole.

Results and Conclusions

- A robust automated high-throughput screening method was developed to assess inhibition of the five major cytochrome P450 enzymes involved in drug metabolism using P450 Baculosomes® and Vivid® fluorogenic substrates.
- Reagent stability, assay linearity, and fluorescent signal stability were key factors that enabled development of a robust automation method. Assay compatibility with 1% DMSO enabled use of a standardized compound storage and handling system.
- The Vivid® assays have proven to be robust, with low variability and excellent Z′-factor values (>0.8).
- The suite of Vivid® P450 inhibition assays has been validated with a panel of compounds with known effects on P450-mediated metabolism. Results were in agreement with reported P450 interactions of these drugs.
- Using this method, a P450 inhibition profile can be generated for any novel test compound both quickly and with a high degree of confidence. This service is available from Invitrogen on a custom basis with a rapid project turnaround time.