Screening Protocol and Assay Conditions

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Assay Theory

Life Technologies' SelectScreen P450 Profiling Service offers a platform for the assessment of cytochrome P450 inhibition. The assays employed in the service use P450 Baculosomes™ and Vivid™ Substrates in a homogenous, fluorogenic format. P450 Baculosomes are microsomes prepared from insect cells infected with recombinant baculovirus containing cDNA for a single human P450 isozyme and rabbit NADPH-cytochrome P450 reductase. The Vivid Substrates possess a low fluorescence until cleaved by cytochrome P450, whereupon a highly fluorescent metabolite is released. Inhibitors of cytochrome P450 activity are readily identified by their ability to prevent formation of fluorescent signal in the assay.

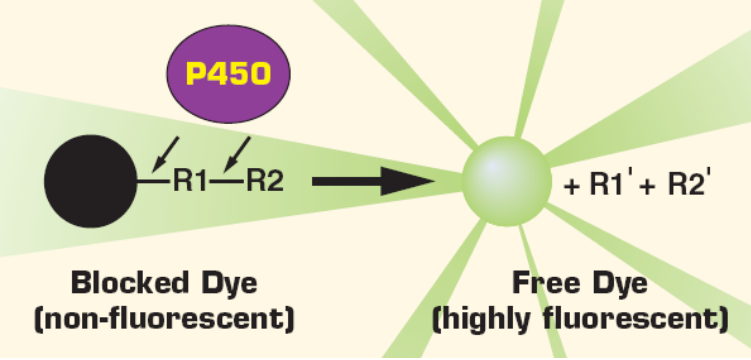

Figure 1 – Vivid Substrates have two potential sites for metabolism (indicated by the arrows), and oxidation at either site by cytochrome P450 converts the blocked, non-fluorescent dye into a highly fluorescent metabolite.

Test compounds are first incubated with P450 Baculosomes and Vivid Substrate in the absence of enzyme turnover to allow binding to reach equilibrium. The reaction is initiated by addition of an NADPH Regeneration System, which supplies a constant amount of the cofactor NAPDH. At the end of the incubation period, addition of a Stop Solution destroys P450 activity by increasing the pH and results in a stable fluorescent signal. Inhibition is then determined by comparing the fluorescence intensity of wells containing test compound to those containing an appropriate solvent control.

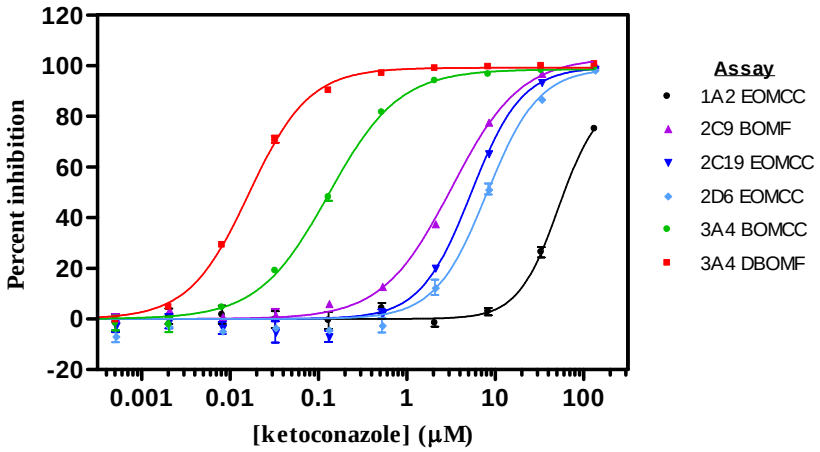

Figure 2 – Example P450 Profiling data using ketoconazole, a known selective CYP3A inhibitor.

SelectScreen Assay Conditions

Test Compounds

All Test Compounds are initially prepared at a 100X concentration in 100% DMSO. The 100X concentration is then diluted to a 3X working concentration in 100 mM potassium phosphate pH 7.4. The Test Compounds are in 1% DMSO final in the well.

P450/Substrate and Control/Substrate Mixtures

The P450/Substrate Mixtures are diluted to a 3X working concentration as indicated in the table below. An appropriate Control/Substrate Mixture is prepared by substituting an amount of control Baculosomes in place of the P450 Baculosomes at an equivalent microsomal protein concentration.

P450/Substrate	3X Mixture Concentration			1X Final Concentration in Assay		
	[P450] (nM)	[Substrate] (µM)	Potassium Phosphate pH 7.4 (mM)	[P450] (nM)	[Substrate] (µM)	Potassium Phosphate pH 7.4 (mM)
1A2 EOMCC	15	9	100	5	3	100
2B6 BOMCC	15	9	100	5	3	100
2C8 DBOMF	15	3	50	5	1	83
2C9 BOMF	30	3	50	10	1	83
2C19 EOMCC	15	30	100	5	10	100
2D6 EOMCC	30	30	100	10	10	100
2J2 MOBFC	15	9	50	5	3	83
3A4 BOMCC	7.5	30	100	2.5	10	100
3A4 DBOMF	7.5	6	100	2.5	2	100
3A5 BOMCC	15	30	100	5	10	100
3A5 DBOMF	15	6	100	5	2	100

NADPH Regeneration Mixture

A 3X working solution of the NADPH Regeneration Mixture is prepared by diluting $NADP^+$ (P2879) to a concentration of 90 µM and 100X Regeneration System (P2878) to a 3X concentration in 100 mM potassium phosphate pH 7.4. The final 1X concentration of these components in the assay is 30 µM $NADP^+$, 3.33 mM glucose-6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase.

Stop Solution

The stop solution contains 0.5 M tris base pH 10.5.

Assay Protocol

1. 5 µL of the 3X Test Compound Solution is added to a low volume 384-well assay plate (Corning Cat. #4511).
2. 5 µL of the 3X P450/Substrate Mixture or 3X Control/Substrate Mixture is added to the plate.
3. Assay plate is shaken on a plate shaker for 30 seconds.
3. Assay plate is incubated for 15-25 minutes at room temperature.
4. 5 µL of the NADPH Regeneration Mixture is added to the plate.
5. Assay plate is shaken on a plate shaker for 30 seconds.
6. Assay plate is incubated for 20-30 minutes at room temperature.

7. 5 µL of the Stop Solution is added to the plate.
8. Assay plate is shaken on a plate shaker for 30 seconds.
9. Assay plate is read on a fluorescence plate reader and the data is analyzed.

SelectScreen Assay Controls

The following controls are made for each individual assay, on every assay plate:

0% Inhibition Control
The maximum fluorescence intensity in a screen, representing the amount of fluorescent metabolite formed by the P450 in the absence of inhibitor, is established by the 0% Inhibition Control. These control wells contain 1% DMSO, P450/Substrate Mixture, and NADPH Regeneration Mixture during the assay incubation.

100% Inhibition Control
The minimum fluorescence intensity in a screen, representing the fluorescence of the assay components in the absence of P450 activity, is established by the 100% Inhibition Control. These control wells contain 1% DMSO, Control/Substrate Mixture, and NADPH Regeneration Mixture during the assay incubation.

Known Inhibitor
A known inhibitor control standard curve, 8-point titration, is run for each individual P450 on the same plate as the corresponding screen with that P450 to ensure that the reaction is inhibited within an expected IC_{50} range.

The following control is prepared for each concentration of Test Compound assayed:

Test Compound Interference (TCI)
Test Compound Interference is evaluated by comparing the fluorescence intensity of wells containing Test Compound, Control/Substrate Mixture, and NADPH Regeneration Mixture during the assay incubation to that of wells containing 1% DMSO, Control/Substrate Mixture, and NADPH Regeneration Mixture during the assay incubation.

SelectScreen Data Analysis

The following equations are used for each set of data points:

	Equation
% Inhibition	$\left\{ 1 - \frac{\mathbf{FI}_{\text{Sample}} - \mathbf{FI}_{\text{TCFI}}}{\mathbf{FI}_{\text{0\% Inhibition}} - \text{FI}_{\text{100\% Inhibition}}} \right\} * 100$
Z´-Factor	$1 - \frac{\mathbf{3*Stdev}_{\text{0\% Inhibition}} + \mathbf{3*Stdev}_{\text{100\% Inhibition}}}{\mathbf{Mean}_{\text{0\%Inhibition}} - \mathbf{Mean}_{\text{100\% Inhibition}}}$
Test Compound Interference Flagged if the value is ≥20%	$\left\{ \frac{\mathbf{FI}_{\text{TCFI}} - \mathbf{FI}_{\text{100\% Inhibition}}}{\mathbf{FI}_{\text{0\% Inhibition}} - \text{FI}_{\text{100\% Inhibition}}} \right\} * 100$

FI $_{\textbf{Sample}}$ = Fluorescence intensity of sample well
FI $_{\text{0\% Inhibition}}$ = Average fluorescence intensity of 0% Inhibition control wells
FI$_{\text{100\% Inhibition}}$ = Average fluorescence intensity of 100% Inhibition control wells
TCI = Test Compound Interference control

Graphing Software

The P450 Profiling Service uses XL*fit* from IDBS. The dose response curve is fit to model number 205 (sigmoidal dose-response model). The bottom of the curve is fit between -20% and 20% inhibition and the top of the curve is fit between 80% and 110%.

P450-Specific Assay Conditions

CYP1A2
The 3X CYP1A2 / EOMCC Mixture is prepared in 100 mM potassium phosphate pH 7.4. The final 15 µL reaction consists of 5 nM CYP1A2 and 3 µM EOMCC in 100 mM potassium phosphate pH 7.4 with 30 µM $NADP^+$, 3.33 mM glucose-6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase. After the reaction incubation, 5 µL of 0.5 M tris pH 10.5 is added.

CYP2B6
The 3X CYP2B6 / BOMCC Mixture is prepared in 100 mM potassium phosphate pH 7.4. The final 15 µL reaction consists of 5 nM CYP2B6 and 3 µM BOMCC in 100 mM potassium phosphate pH 7.4 with 30 µM $NADP^+$, 3.33 mM glucose-6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase. After the reaction incubation, 5 µL of 0.5 M tris pH 10.5 is added.

CYP2C8
The 3X CYP2C8 / DBOMF Mixture is prepared in 50 mM potassium phosphate pH 7.4. The final 15 µL reaction consists of 5 nM CYP2C8 and 1 µM BOMF in 83 mM potassium phosphate pH 7.4 with 30 µM $NADP^+$, 3.33 mM glucose-6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase. After the reaction incubation, 5 µL of 0.5 M tris pH 10.5 is added.

CYP2C9
The 3X CYP2C9 / BOMF Mixture is prepared in 50 mM potassium phosphate pH 7.4. The final 15 µL reaction consists of 10 nM CYP2C9 and 1 µM BOMF in 83 mM potassium phosphate pH 7.4 with 30 µM $NADP^+$, 3.33 mM glucose-6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase. After the reaction incubation, 5 µL of 0.5 M tris pH 10.5 is added.

CYP2C19
The 3X CYP2C19 / EOMCC Mixture is prepared in 100 mM potassium phosphate pH 7.4. The final 15 µL reaction consists of 5 nM CYP2C19 and 10 µM EOMCC in 100 mM potassium phosphate pH 7.4 with 30 µM $NADP^+$, 3.33 mM glucose-6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase. After the reaction incubation, 5 µL of 0.5 M tris pH 10.5 is added.

CYP2D6
The 3X CYP2D6 / EOMCC Mixture is prepared in 100 mM potassium phosphate pH 7.4. The final 15 µL reaction consists of 10 nM CYP2D6 and 10 µM EOMCC in 100 mM potassium phosphate pH 7.4 with 30 µM $NADP^+$, 3.33 mM glucose-6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase. After the reaction incubation, 5 µL of 0.5 M tris pH 10.5 is added.

CYP2J2
The 3X CYP2J2 / MOBFC Mixture is prepared in 50 mM potassium phosphate pH 7.4. The final 15 µL reaction consists of 5 nM CYP2J2 and 3 µM MOBFC in 83 mM potassium phosphate pH 7.4 with 30 µM $NADP^+$, 3.33 mM glucose-6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase. After the reaction incubation, 5 µL of 0.5 M tris pH 10.5 is added.

CYP3A4 (BOMCC)
The 3X CYP3A4 / BOMCC Mixture is prepared in 100 mM potassium phosphate pH 7.4. The final 15 µL reaction consists of 2.5 nM CYP3A4 and 10 µM BOMCC in 100 mM potassium phosphate pH 7.4 with 30 µM $NADP^+$, 3.33 mM glucose-6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase. After the reaction incubation, 5 µL of 0.5 M tris pH 10.5 is added.

CYP3A4 (DBOMF)
The 3X CYP3A4 / DBOMF Mixture is prepared in 100 mM potassium phosphate pH 7.4. The final 15 µL reaction consists of 2.5 nM CYP3A4 and 2 µM DBOMF in 100 mM potassium phosphate pH 7.4 with 30 µM $NADP^+$, 3.33 mM glucose-6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase. After the reaction incubation, 5 µL of 0.5 M tris pH 10.5 is added.

CYP3A5 (BOMCC)
The 3X CYP3A5 / BOMCC Mixture is prepared in 100 mM potassium phosphate pH 7.4. The final 15 µL reaction consists of 5 nM CYP3A5 and 10 µM BOMCC in 100 mM potassium phosphate pH 7.4 with 30 µM $NADP^+$, 3.33 mM glucose-6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase. After the reaction incubation, 5 µL of 0.5 M tris pH 10.5 is added.

CYP3A5 (DBOMF)
The 3X CYP3A5 / DBOMF Mixture is prepared in 100 mM potassium phosphate pH 7.4. The final 15 µL reaction consists of 5 nM CYP3A5 and 2 µM DBOMF in 100 mM potassium phosphate pH 7.4 with 30 µM $NADP^+$, 3.33 mM glucose-6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase. After the reaction incubation, 5 µL of 0.5 M tris pH 10.5 is added.

TABLE OF P450 INHIBITOR VALIDATION

The table below provides IC_{50} values (nM) for P450 inhibitors.

Assay	Known Inhibitor	IC50 [nM]
1A2 EOMCC	α-naphthoflavone	16
2B6 BOMCC	Miconazole	126
2C19 EMOCC	Miconazole	69
2C8 DBOMF	montelukast	100
2C9 BOMF	Sulfaphenazole	114
2D6 EOMCC	Quinidine	5
2J2 MOBFC	terfenadine	300
3A4 BOMCC	Ketoconazole	45
3A4 DBOMF	Ketoconazole	6.9
3A5 BOMCC	Ketoconazole	71
3A5 DBOMF	Ketoconazole	30