



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.

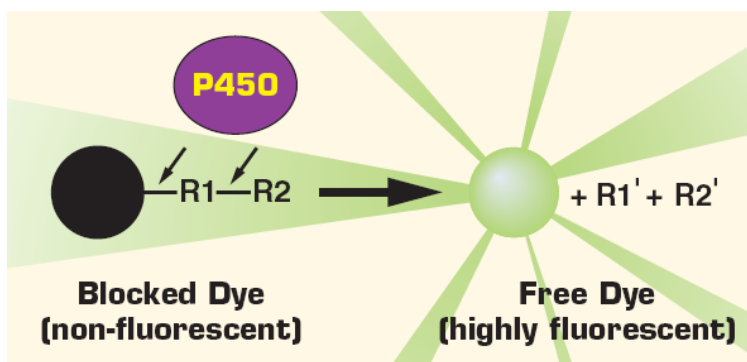


Figure 1 – Vivid Substrates have two potential sites for metabolism (indicated by the arrows), and oxidation at either site by cytochrome P450 converts the blocked, non-fluorescent dye into a highly fluorescent metabolite.

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 NADPH. 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.

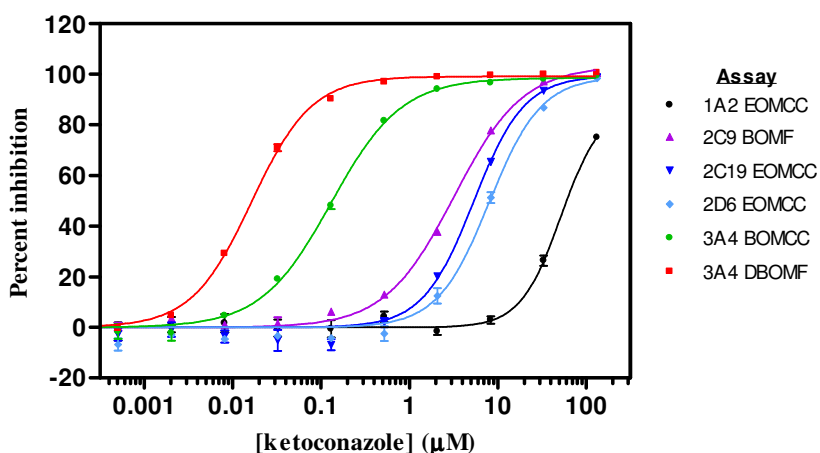


Figure 2 – Example P450 Profiling data using ketoconazole, a known selective CYP3A inhibitor.

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 Mixture Concentration			Known Inhibitor	IC50 (nM)
	[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	a-naphthoflavone	16
2B6 BOMCC	15	9	100	5	3	100	Miconazole	126
2C19 EOMCC	15	30	50	5	10	50	Miconazole	69
2C8 DBOMF	15	3	50	5	1	50	Montelukast	100
2C9 BOMF	30	3	50	10	1	50	Sulfaphenazole	114
2D6 EOMCC	30	30	100	10	10	100	Quinidine	5
2J2 MOBFC	15	9	50	5	30	50	Terfenadine	300
3A4 BOMCC	7.5	30	100	2.5	10	100	Ketoconazole	9.8
3A4 DBOMF	7.5	6	100	2.5	2	100	Ketoconazole	1.27
3A5 BOMCC	15	6	100	5	2	100	Ketoconazole	38
3A5 DBOMF	15	6	100	5	2	100	Ketoconazole	30

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

- 5 μL of the 3X Test Compound Solution is added to a low volume 384-well assay plate (Corning Cat. #4511).
- 5 μL of the 3X P450/Substrate Mixture or 3X Control/Substrate Mixture is added to the plate.

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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 μ L 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 μ L 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 IC₅₀ 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	$\left\{ 1 - \frac{\mathbf{FI}_{\text{Sample}} - \mathbf{FI}_{\text{TCFI}}}{\mathbf{FI}_{0\% \text{ Inhibition}} - \mathbf{FI}_{100\% \text{ Inhibition}}} \right\} * 100$
Z'-Factor	$1 - \frac{3 * \text{Stdev}_{0\% \text{ Inhibition}} + 3 * \text{Stdev}_{100\% \text{ Inhibition}}}{\text{Mean}_{0\% \text{ Inhibition}} - \text{Mean}_{100\% \text{ Inhibition}}}$
Test Compound Interference Flagged if the value is $\geq 20\%$	$\left\{ \frac{\mathbf{FI}_{\text{TCFI}} - \mathbf{FI}_{100\% \text{ Inhibition}}}{\mathbf{FI}_{0\% \text{ Inhibition}} - \mathbf{FI}_{100\% \text{ Inhibition}}} \right\} * 100$

FI_{Sample} = Fluorescence intensity of sample well

FI_{0% Inhibition} = Average fluorescence intensity of 0% Inhibition control wells

FI_{100% Inhibition} = Average fluorescence intensity of 100% Inhibition control wells

TCI = Test Compound Interference control

Graphing Software

The P450 Profiling Service uses *XLfit* 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%.