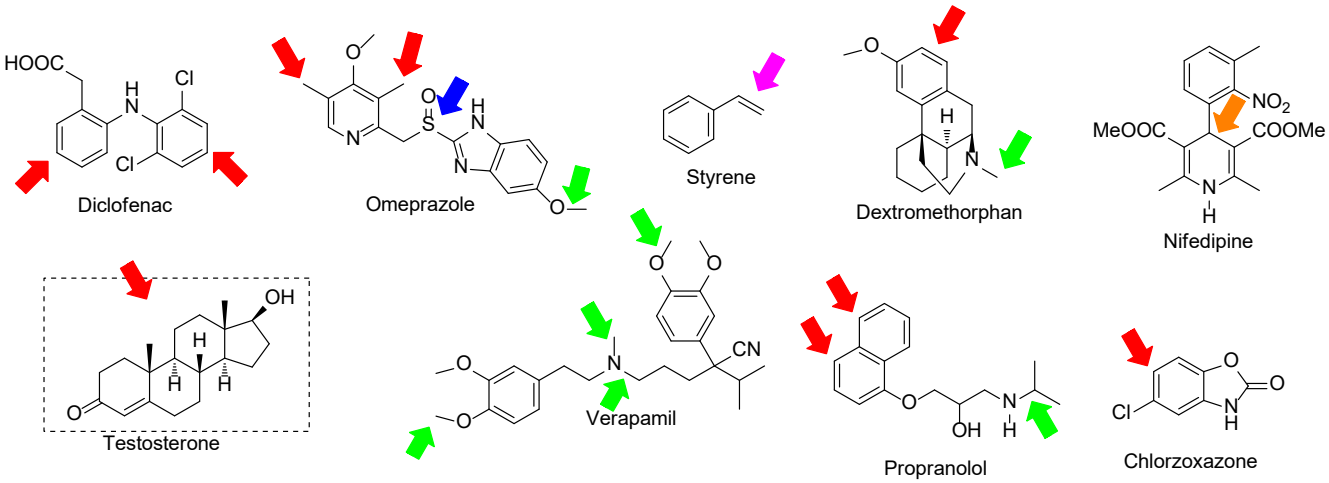
Codex® MicroCyp® Screening Kits

Screening Protocol

REACTIVITY EXAMPLES



hydroxylation / ***O*- and *N*-dealkylation** / **heteroatom oxidation** / **dehydrogenation** / **epoxidation**

BACKGROUND INFORMATION

The Codex® MicroCyp® (MCYP®) Screening Kits contain engineered cytochrome P450 (CYP) variants of CYP102A1 from *Bacillus megaterium* (BM3) that have been evolved to produce mammalian-type metabolites. Our MicroCyp® enzymes show broad substrate specificity, are expressed at high levels in their bacterial host and have shown up to 100-fold increases in productivity when compared with human CYP activity. Combined with their lower cost of manufacture, MicroCyp® enzymes can produce gram quantities of metabolites cost effectively. In addition, the MicroCyp® enzymes are capable of other types of transformations that can be useful in lead diversification programs.

The MicroCyp® Screening Kits are provided with all the necessary reagents and protocols to enable rapid identification of the enzyme(s) producing the compound of interest. The Screening Kits are arrayed in a 24-well format, suitable for manual or robotic manipulation. Each individual MicroCyp® enzyme is available as lyophilized enzyme powder for scale up synthesis of the desired product.

KIT INFORMATION

Recommended storage temperature is −20 °C when stored for a week, and −80 °C when stored for longer period. The Standard MicroCyp® Screening Kit contains 23 standard enzymes while the Elite MicroCyp® Screening Kit contains 11 highly improved enzymes in duplicate wells for the ability to screen two compounds per kit. The Elite MicroCyp® Screening Kit has improved activity, greater substrate promiscuity and increased solvent tolerance.

24-WELL KIT SCREENING PROCEDURE

Materials & Reagents

1. 24-well MicroCyp® Screening Kit, Standard or Elite Kit (included)
2. MicroCyp® Reaction Mix (MCYP-RXN BUFFER, 1.65 g included)
3. Water (47 mL), cosolvent (acetonitrile or methanol 1.5 mL)
4. Compound

Screening Procedure

1. Thaw the MicroCyp® 24-well plate at room temperature for 10–20 min.
2. Dissolve 1.65 g MCYP-RXN BUFFER in 46.6 mL de-ionized water and if necessary, adjust the pH to 8.0 with 1 M KOH.
3. Dissolve compound in 1.4 mL acetonitrile, methanol or water.
4. Add the compound solution to the MCYP-RXN BUFFER solution and bring the total volume to 48 mL.
5. Re-check the pH, and if necessary, re-adjust to 8.0 with 1 M KOH, 1 M H_3PO_4 or 1 M HCl.
6. Use a multi-channel pipette to add 2.0 mL of the final MCYP-RXN BUFFER solution to each well.
7. Place the plate with its cover top in a shaker oven with 85% humidity at 30 °C at 250 RPM for 4–16 hours.
   Note: a temperature range between 25 and 35 °C is acceptable, with 30 °C as the optimal temperature.

Reagent Concentration	Volume per Well	Volume per Plate	Final Concentration
Enzyme solution (6 µM)	400 µL	9.6 mL	16.7% v/v, 1 µM
MCYP-RXN BUFFER solution • 0.3–2.4 mM compound • ≤3.0% cosolvent • 1.2 mM $NADP^+$ • 30 mM Glucose • 0.6 mg/mL Glucose Dehydrogenase • 120 mM potassium phosphate buffer, pH 8.0	2.0 mL	48 mL	83.3% v/v • 0.25–2.0 mM compound • ≤2.5% cosolvent • 1 mM $NADP^+$ • 25 mM Glucose • 0.5 mg/mL Glucose Dehydrogenase • 100 mM potassium phosphate buffer, pH 8.0
Total Volume	2.4 mL	57.6 mL	

Work-Up & Analysis

1. To stop the reaction, add 2.4 mL of a suitable organic solvent (acetonitrile, methanol) to each well, seal the entire plate carefully and agitate at 200 rpm for 10 min at room temperature to ensure protein precipitation.
2. Centrifuge the plate at 3,000–5,000 rpm for 15 min to pellet insoluble materials. Alternatively, filtration through a 2-micron filter can be used.
3. Transfer 100 µL of the supernatant from each well to a shallow well plate and seal the plate (when using a heat-sealer set at 180 °C for 3 sec).
4. Analyze each sample by preferred method of analysis.

5. The plate layout can be found on page 4 (Appendix I).

USEFUL TIPS

If the substrate is not soluble in water, make a stock solution in acetonitrile or methanol. If substrate solubility is not improved using acetonitrile or methanol, make a stock solution in DMSO with a final DMSO concentration of no more than 5% v/v. However, most Elite MYCPs can tolerate organic solvent concentrations up to 10% v/v. An inverse relationship between activity and organic solvent concentration is generally observed for all enzymes.

Previous studies have shown that certain water-soluble substrates may benefit from adding 1% acetonitrile. Do not sonicate any solution containing MCYP-RXN BUFFER. This mix contains Glucose Dehydrogenase, an enzyme required for NADPH recycling which will be inactivated when sonicated.

NOTES

- Some enzymes may lose activity using DMSO or increased solvent concentrations. It is recommended that initial experiments are carried out to determine the range of substrate solubility in water and/or mixed solvent systems (*i.e.* DMSO or other organic solvent with dissolved substrate mixed with water) before running a reaction with the MicroCyp® Screening Kit.

- The MCYP-RXN BUFFER solution containing $NADP^+$, GDH and substrate should be prepared just before use.

- The recommended final concentration of compound should be between 0.25–2.0 mM. If a very low concentration is used (20–50 µM), the reaction time can be shortened to 30 min–2 hrs.

- The reaction volume should not exceed the indicated volume per well since larger volumes result in poor aeration of the reaction and lower yields. Agitation of at least 250 rpm is important for sufficient oxygen transfer.

HOW TO DETERMINE HOW MUCH MICROCYP IS REQUIRED FOR SCALE UP

The MicroCyp® enzyme conversions usually scale up very linearly. Doing a calculation of productivity using the molecular mass, concentration, % conversion and the enzyme concentration (1 µM), one can determine the volume at which to run the large-scale reaction to achieve the desired amount of product.

As an example, assume the following:

- The parent drug (mass: 350) is screened at a concentration of 0.5 mM.
- After 4 h, we find 42% conversion to the desired hydroxylated metabolite of +16.
- We need to have 15 mg pure metabolite for further studies.

In this case, the productivity is 0.42 x 0.5 x 366 = 76.9 mg/L. To allow for some losses in purification, one should aim to produce ~30 mg crude metabolite. This would result in a scale up volume of 30/76.9 = 390 mL. To achieve a 1 µM enzyme concentration, 390 nmol MicroCyp® enzyme in 390 mL is required.

LITERATURE

- J. de Vlieger, *et al*. Determination and identification of estrogenic compounds generated with biosynthetic enzymes using hyphenated screening assays, high resolution mass spectrometry and off-line NMR. *J. Chromatogr. B.* **2010**, *878*, 667-674.

- F. H. Arnold, *et al*. A Panel of Cytochrome P450 BM3 Variants to Produce Drug Metabolites and Diversify Lead Compounds. *Chem. Eur. J.* **2009**, *15*, 11723-11729.

- F. H. Arnold, *et al.* Preparation of human metabolites of propranolol using laboratory-evolved bacterial cytochromes P450. *Biotech. Bioeng.* **2006**, *93*, 494-499.

- M. A. Hayes, *et al*. CYP3A Specifically Catalyzes 1*β*-Hydroxylation of Deoxycholic Acid: Characterization and Enzymatic Synthesis of a Potential Novel Urinary Biomarker for CYP3A Activity. *Drug Metabolism & Disposition.* **2016**, *44,* 1480-1489.

APPENDIX I: 24-WELL KIT LAYOUT

This is a reference for the MicroCyp® Screening Kits to aid in identifying your hits and for purchasing individual MicroCyp® Enzymes. When placing an order for individual enzymes, please provide the Enzyme Order Number.

MCYP-0361 KIT (Standard Kit)			MCYP-0363 KIT (Elite Kit)		
#	Well	Enzyme Order Number	#	Well	Enzyme Order Number
1	A01	MCYP0009	1	A01	MCYP0130
2	A02	MCYP0015	2	A02	MCYP0139
3	A03	MCYP0027	3	A03	MCYP0141
4	A04	MCYP0029	4	A04	MCYP0143
5	A05	MCYP0030	5	A05	MCYP0145
6	A06	MCYP0032	6	A06	MCYP0148
7	B01	MCYP0034	7	B01	MCYP0150
8	B02	MCYP0035	8	B02	MCYP0151
9	B03	MCYP0057	9	B03	MCYP0153
10	B04	MCYP-P1.2-A07	10	B04	MCYP-P1.2-A07
11	B05	BLANK	11	B05	MCYP0155
12	B06	MCYP-P1.2-A05	12	B06	MCYP0156
13	C01	MCYP-P1.2-A12	13	C01	MCYP0130
14	C02	MCYP-P1.2-B10	14	C02	MCYP0139
15	C03	MCYP-P1.2-B11	15	C03	MCYP0141
16	C04	MCYP-P1.2-B12	16	C04	MCYP0143
17	C05	MCYP-P1.2-D07	17	C05	MCYP0145
18	C06	MCYP-P1.2-D09	18	C06	MCYP0148
19	D01	MCYP0002	19	D01	MCYP0150
20	D02	MCYP0005	20	D02	MCYP0151
21	D03	MCYP0013	21	D03	MCYP0153
22	D04	MCYP0014	22	D04	BLANK
23	D05	MCYP0016	23	D05	MCYP0155
24	D06	MCYP0052	24	D06	MCYP0156

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