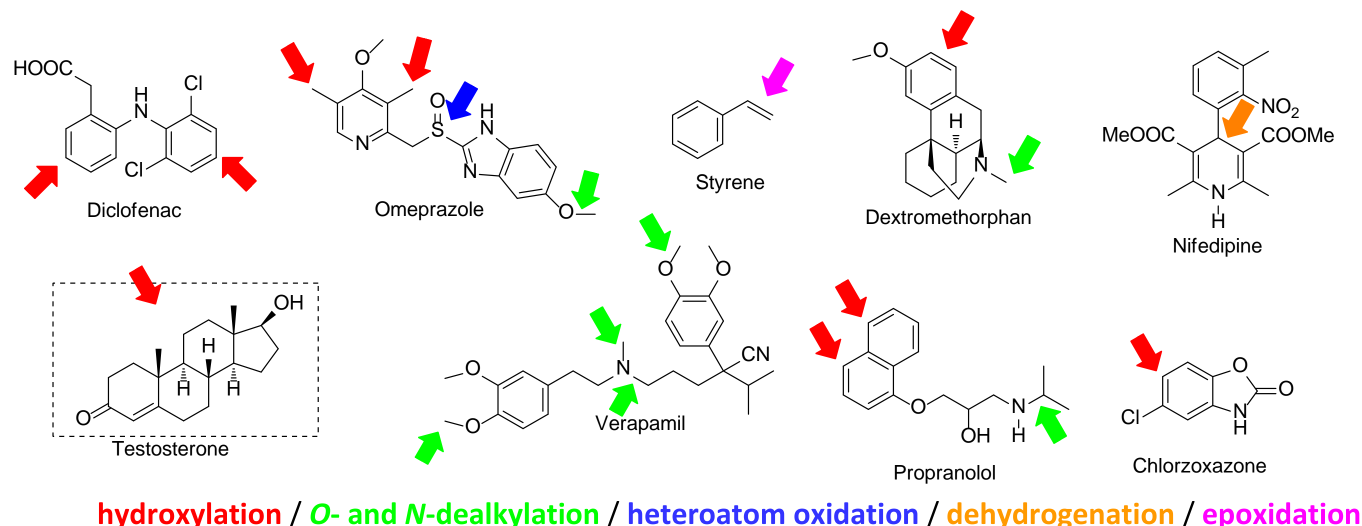
Codex® MicroCyp® Screening Kits

Screening Protocol

REACTIVITY EXAMPLES



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

Codexis, Inc.
200 Penobscot Drive, Redwood City, CA 94063 USA
www.codexis.com | sales@codexis.com | Phone: +1 (650) 421-8100

Codex® MicroCyp® Screening Kits

Frequently Asked Questions

OVERVIEW

What is the MicroCyp® Screening Kit?

The 24-well MicroCyp® Screening Kits contain engineered P450 enzymes that offer a simple approach to functionalize compounds for mammalian-type metabolite production or lead diversification.

The Standard MicroCyp® Screening Kit (catalog # MCYP-0361 KIT) contains 23 enzymes and allows for one compound screen per kit while the Elite MicroCyp® Screening Kit (catalog # MCYP-0363 KIT) contains 11 improved enzymes in duplicate wells to allow for two compound screens per kit. The Elite MicroCyp® enzymes are greatly improved over the Standard MicroCyp® enzymes – they are more active, have wider substrate promiscuity and solvent tolerance.

What is the enzyme concentration in each well and what is the working volume?

- The starting enzyme concentration in each well is 6 µM and the final concentration is 1 µM. The total working volume per well is 2.4 mL.
- The 24-well kit working volume allows for generation of larger absolute amounts of metabolite that is often enough for NMR analysis.
- If a smaller scale reaction is desired or if you need to do multiple screens, you may aliquot the enzyme solution out of the 24-well panels to use in your smaller, or separate reaction.

What are the variants in the kit and where are the controls?

- See the Appendix for the plate layout. The control well contains enzyme MCYP-P1.2-A07. Additionally, there is also a "Blank" (buffer only) well in each panel.

Will the MicroCyp® enzymes make human metabolites?

- They often do. We have seen a good hit-rate but in some cases, human CYPs work better. An advantage of the MCYP® enzymes is they often give metabolites that are not formed by the human CYPs and this can be very useful in areas such as lead diversification.

Do the MicroCyp® enzymes correlate directly with the human CYPs, *i.e.* does one MicroCyp® enzyme have the exact same activity as for instance CYP3A4?

- In general, no. The enzymes in the MicroCyp® Screening Kits are variants of bacterial CYPs and there is a significant structural difference around the active site between the MCYP® enzymes and human CYPs. It is therefore not guaranteed that you get the exact same activity.

How long can I store the kits and at what temperature should the kits be stored?

- The MicroCyp® Screening Kits are stable for at least one year at −80 °C and several months at −20 °C. It is best to avoid freeze-thaw cycles since that can cause a decrease in enzyme activity.

How do you assay the active MicroCyp® enzyme concentration in each well?

- The quantification of active P450 enzyme present is performed by a CO-binding reduced difference assay. P450 enzymes (of which MCYP® enzyme belong) contain a heme-thiolate bond which, upon reduction by dithionate and binding of CO, results in a shift of the heme Soret band to 450 nm (hence the name). If the heme-thiolate bond is broken, representing inactive degraded enzyme, the Soret band remains at 420 nm. An increase in absorbance at 450 nm indicates a catalytically active enzyme. The assay is performed at 22 ^{0}C in the presence of carbon monoxide and 17.3 mM sodium dithionate at pH 8.0.

PLANNING YOUR SCREENING EXPERIMENT

Does the kit contain positive and negative controls?

- We use enzyme MCYP-P1.2-A07 as positive controls in both 24-well kits. The negative control contains buffer only.

What are in the negative control wells?

- The negative controls are wells containing no MicroCyp® enzyme. Thus, they will have a similar background contamination in the LC-MS (cell components, media components, etc.) and serve as a blank for analytical verification.

Should the test compound be added to the replicates and negative wells?

- Yes, it is recommended that you add the test compound to all wells. Doing so will allow you to verify recovery of parent compound in the negative control wells and can pinpoint potential loss of material in the matrix. In the ideal case, you would dispense the parent compound in only some of the negative control-wells. Those without the parent compound would allow you to determine if any background peaks on the LC-MS are due to the matrix.

What if no new or expected product peaks are observed after incubating the parent compound on the MicroCyp® Screening Kit?

- If it is a new, untested compound, it is possible that none of the tested MCYP® enzymes are active towards it. If you are screening only the Standard MicroCyp® Kit, it is recommended that you screen the Elite MicroCyp® as well.

- A positive control, such as testosterone or diclofenac can be tested in wells containing MCYP-P1.2-A07, as these are known to be good substrates for this MicroCyp® enzyme.

What pH should I perform the reactions at?

- For all kits and individual powders, the pH will be ~8.0 when the supplied buffer mix is used, unless a very acidic or basic substrate is added. But it is recommended to check the pH of the buffer mix/compound solution before adding it to the enzymes in the 24-well plates. In general, the pH range of the MicroCyp® enzymes is somewhat narrow, so it is important that the pH does not deviate significantly from pH 8.0.

Which co-solvents can I use and what is the upper limit of co-solvent concentration in the system?

- For co-solvents we recommend using no more than 5% of a water miscible solvent such as methanol, ethanol, isopropanol, acetonitrile, DMSO or acetone. We have had the best results when using methanol or acetonitrile. It is recommended that initial experiments are performed to determine the range of substrate solubility in water and/or mixed solvent systems before running a reaction with the MicroCyp® Screening Kit. Minimizing co-solvent concentration, while maintaining substrate solubility, is preferred as higher co-solvent concentration may result in lower enzyme activity and hydroxylation of the co-solvent itself.

What substrate concentration range should I use?

- Each substrate will be different but generally a first screen should be performed with concentrations below 0.75 mM; a good starting point is 0.5 mM. If poor results are obtained initially, this could be due to substrate and/or product inhibition, thus reducing the substrate concentration could partially alleviate this.

COMMON SCREENING ISSUES

My substrate is not very soluble, what can I do?

- Try a different solvent. For some substrates, acetonitrile results in better solubility.

- Try adding the substrate from a stock solution directly to the plate, instead of mixing it in with the buffer mix as the protocol states, as this could result in better dispersion of the compound.

Can the reactions be run in vials with stir bars?

- We recommend running the reactions in the plate provided with the enzymes. These plates have baffling at the base of the wells that aids in sample mixing and oxygen transfer. Good oxygen transfer is essential for optimal conversion.

What can be done if the LC-MS column is clogging?

- This is likely due to residual protein fragments in the sample. It is recommended to spin down the plates or samples in a centrifuge for 15 min at 4,000 RPM at 4 °C. This is usually sufficient to pellet out most insolubles. If your centrifuge cannot cool the sample, it may be necessary to centrifuge for an additional 15 min to ensure good pelleting of the insoluble.

- Extraction or quenching with 2 reaction volumes of an organic solvent, followed by centrifugation will also minimize particulates from entering the column.

- An injection volume of 2–10 µL is recommended, and the column should be protected by a guard column. The guard column should last about 100 injections before needing replacement.

SCALE-UP

What if lower conversion is achieved when scaling-up the reaction?

- A change in conversion during scale-up is not uncommon. The MCYP® enzymes require oxygen transfer and typically oxygen transfer during scale-up is reduced due to poor mixing. Scaling-up in a regular flask is usually not sufficient. Baffled flasks with vigorous shaking will maximize oxygen transfer and conversion. Other aspects of the reaction such as temperature, pH and co-solvent type and its concentration should then be investigated to further optimize product yield.

How much enzyme is needed to scale-up the reaction?

- These reactions typically scale-up linearly. A calculation can be performed to determine what volume the larger scale reaction should be performed at to achieve the desired amount of product. An example is the following:
  - The parent drug (molecular mass of 350 Da) was screened at a concentration of 0.5 mM.
  - After 4 h, there is 42% conversion to the desired hydroxylated metabolite of +16 (366 Da).
  - For further studies, 15 mg of pure metabolite is required.
  - In this case, the product concentration is 77 mg/L (0.42 x 0.5 x 366). To allow for some losses in purification, aim to produce ~30 mg crude metabolite. This would require a scale-up volume of 390 mL (30 mg/0.077 mg/mL). To

perform this reaction with the same 1 µM enzyme concentration, this would require 390 nmol of the MicroCyp® in the 390 mL reaction volume.

What can be done to maximize the conversion at larger scale?

- There are several parameters of the reaction that can be investigated to improve the reaction conversion:
  - Vary the parent compound concentration.
  - Vary the reaction time.
  - Increase the enzyme concentration (1 µM vs. 2.5 µM vs. 5 µM).
  - Use an enzyme dosing strategy.
  - Vary the reaction temperature or pH.

- **Other considerations:**

  - In some cases, it may be preferable to have poorer conversion but perform the reaction at a higher parent compound concentration. In this case, while the conversion may be low, the total amount of metabolite produced may be higher and thus more is available for subsequent analysis or testing.
  - In cases where it is difficult to separate the metabolite from the parent compound, it may be preferable to perform the reaction at a lower parent compound concentration such that the conversion is high and most of it is converted to the metabolite.

Who do I contact if I have further questions?

- Please contact your Business Development account manager directly, or email us at sales@codexis.com for technical support or general questions.