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 ^{o}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.