Protocol for Human P450 Metabolite Identification & Production

PROCEDURAL NOTES

- Unlike older *in vitro* P450 systems, *CypExpress*™ does NOT require addition of glucose 6-phosphate dehydrogenase (G6PDH) or magnesium for activity. In fact, certain metals, such as magnesium and calcium may complex with some substrates – thereby reducing solubility and metabolite production.

- Substrate concentrations up to 3.0 mM have been used with *CypExpress*™. Typically we use 50-200 µM for P450 profiling and metabolite identification or 500 µM-1 mM for scale-up metabolite synthesis. The concentration that produces the highest concentration of metabolites varies for each compound and can be optimized using the Pilot Procedure (see below).

- Small-volume reactions can be performed in 24-well microplates provided that there is sufficient agitation for optimal aeration.

- Many drugs are poorly water-soluble and will be difficult to dissolve in buffer. This can be remedied by preparing a concentrated solution of the drug in dimethylsulfoxide or *N,N*-dimethylformamide and adding it to the buffer. DMSO & DMF are the preferred solvents, with a final concentration (v/v) preferably 2% or less in the final reaction mixture. Methanol (o.5-1.0% v/v) may be used, but even at low concentrations may strongly inhibit the activity of some P450 isoforms.

- A 100 mg/mL concentration of *CypExpress*™ is typically best for P450 profiling and metabolite identification. The concentration of *CypExpress*™ for Scale-up metabolite synthesis can be lowered for good substrates, especially when employing the Multi-Cycle Procedure.

Materials

- *CypExpress*™ powder
- 100 mM, pH 7.4 potassium phosphate buffer containing:
    - 5.0 mM glucose-6-phosphate (G6P)
    - 2.0 mM nicotinamide adenine dinucleotide phosphate, sodium salt ($NADP^+$)
    - Substrate concentration of 500 µM to 3.0 mM
- Pilot Procedure for Scale-up synthesis:
    - Glass test tubes or flasks with Teflon stir bars
- For P450 Profiling and Metabolite Identification
    - 24 or 48 well microplate and microplate shaker

Pilot Procedure

– Use this method to evaluate reaction conditions and optimize metabolite production –

1. Allow all reagents to come to room temperature.
2. Place the *CypExpress*™ powder into a test tube with a stir bar. A 16 mm by 125 mm tube works well.
3. Add 1.0 mL of buffer containing substrate, G6P and $NADP^+$ to the powder and vortex to make a suspension. Use a glass rod or pipette tip to break-up small pieces and lumps.
4. Stir the uncovered tube at 37°C at a rate fast enough to create a vortex.
5. Allow the reaction to proceed for four hours.
6. Quench the reaction with 1.0 mL of HPLC solvent (e.g. methanol or acetonitrile), and centrifuge it at 6,000×*g* for 10 minutes at room temperature.
7. Remove the supernatant and proceed with metabolite separation and analysis.

Multi-Cycle and Scale-Up Procedure

– Use this method to produce larger quantities of metabolites –

1. Scale-up the Pilot Procedure by multiplying the *CypExpress*™ powder amount and buffer volume by the same factor.
   - For example, 10 g of *CypExpress*™ powder and 100 mL of buffer containing $NADP^+$, G6P and 1.0 mM substrate in a 250 mL flask.
2. Allow all reagents to come to room temperature.
3. Place the *CypExpress*™ powder into the flask containing the stir bar.
4. Add the buffer containing substrate, G6P and $NADP^+$ to the powder and shake to make a suspension. Use a glass rod or spatula to break-up small pieces and lumps.
5. Stir the uncovered flask at 37°C at a rate fast enough to create a vortex.
6. Allow the reaction to proceed for four hours.
7. Centrifuge the suspension at 6,000×*g* for 10 minutes at room temperature.
8. Remove the supernatant containing the metabolites.
9. If metabolite production was low, another cycle may be performed by resuspending the pellet in fresh buffer containing $NADP^+$, G6P and substrate.
10. Repeat steps 4 thru 8 up to three times.
11. Before the final centrifugation, add an equal volume of solvent (e.g. methanol or acetone) to the reaction mixture to extract any metabolites that may have been absorbed into the *CypExpress*™ powder.
12. Combine all supernatant fractions for metabolite separation and isolation.

If you have any questions, please contact us:
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