Overview:

Hypha's PolyCYPs® Screening Kit contains recombinant single-enzymes for the oxidation of organic molecules. The PolyCYPs® enzymes are selected from Hypha's talented microbial biotransformation strains. PolyCYPs® enzymes are capable of hydroxylating a wide range of substrate compounds, both aliphatic C-H (e.g. benzylic -CH, $-CH_2$, $-CH_3$, *tert*-butyl, *iso*-propyl, cyclopentyl/hexyl and linear alkyl moieties), as well as aromatic systems to form phenols and epoxides. De-alkylation of *N*- and *O*-alkyl moieties is also observed.

What's in the box?

- **PolyCYPs® Enzyme vials (blue crimp-lid vials)**: CYP450 enzyme, ferredoxin and ferredoxin reductase
  Isoforms provided in the kit: PolyCYPs® 6 (2 vials), 152, 166, 168, 194, 196, 217, 235
- **Cofactor vial (green crimp-lid vial)**: glucose-6-phosphate (G6P), nicotinamide adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate-dehydrogenase (G6PDH), $MgCl_2$, and potassium phosphate buffer to give pH 7.4
- **Bosentan (1 x silver crimp-lid vial)**: Substrate for positive control
- **Formulant (1 x red crimp-lid vial)**: 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD)

  NB: only use for test compounds with aqueous solubility <0.01mg/ml.
- **24-square well polypropylene plate** : To be used for incubation once the reactions are prepared
- **Self-adhesive gas permeable plate seal**: Permits oxygen exchange during incubation

Step by step protocol (500 µl reactions without HP-β-CD)

1. Remove all vials from the 24-well block and open them. Note: The vials are vacuum-sealed.

During the following steps it is recommended to use an ice bath for the reconstituted enzyme & cofactor components, however this is not essential if performing the reaction preparation within *Ca*.30 minutes

2. Dissolve test compound(s) in appropriate solvent (e.g., water, DMSO, acetonitrile or 2-propanol to make a minimum of 25 µl stock solution at 25 mg/ml (for 0.1 mg/ml final substrate concentration).

3. Add 20 µl of DMSO to the bosentan vial, vortex & keep at room temperature before use.

4. Add 1000 µl of cold high purity H_2O to the cofactor vial, gently mix to dissolve.

5. Without mixing, add a total of 448 µl of cold high purity water to each of the PolyCYPs® enzyme vials, stand for approximately 2 minutes before progressing; this reduces protein aggregation.

6. After the 2 minutes soaking time, **gently** agitate the PolyCYPs® enzyme vials using a pipette until a fine suspension/solution is achieved; **do not sonicate or vortex these solutions** - avoid/minimise formation of bubbles otherwise this will reduce the effectiveness of the enzymes.

7. Dispense 2.0 µl of the test compound solution into each vial of PolyCYPs® enzyme solution, mix gently.

8. Dispense 2.0 µl of the bosentan solution to the extra PolyCYPs® **6** enzyme vial provided, mix gently.

9. Dispense 50 µl of the cofactors solution to all PolyCYPs® vials, including the bosentan positive control reaction.

10. Transfer the contents of each vial to one well each of the 24-well plate; seal the plate with the gas-permeable seal.

11. Incubate for 16-20 hrs with agitation, ideally at 27˚C. **Agitation type & speed are the most influential aspects for successful reactions**; for recommended shaker or stirred formats please refer to page 3. Allow longer incubation times if using lower incubation temperatures.

12. Terminate all reactions by adding 500 µl of acetonitrile to each well (1000-1500 µl can be used to ensure dissolution of more apolar substrates) and mix gently (pipetting or shaking). It is normal to occasionally observe a semi-solid aggregate in the reactions after the incubation period.

13. Centrifuge the 24-well block with reaction extracts *in-situ* or transfer to centrifuge tubes. Centrifuge the samples using at least 1,000 x g for 10 minutes to remove insoluble materials.

14. Transfer supernatants to appropriate vials for analysis. Take usual precautions against residual solids.

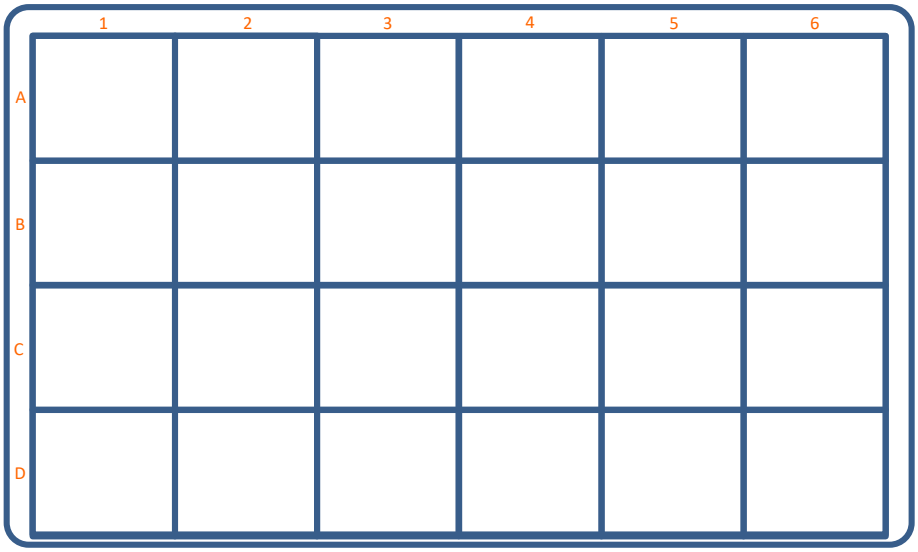
Changes to protocol <u>for substrates of solubility <0.01mg/ml</u> (500 µl reactions with HP-β-CD)

- **Replace step 2 above with:** Dissolve test compound(s) in appropriate solvent (e.g., DMSO, acetonitrile or 2-propanol to make a **minimum of 50 µl stock solution at 25 mg/ml**. Add 40 µl of the test compound solution stock to the **HP-β-CD** vial followed by 460 µl of high purity water. Vortex and keep on the bench until use.
- **In step 5 above change the water volume from 448 µl to 425 µl.**
- **In step 7 above change the test compound solution volume from 2 µl to 25 µl of formulated compound stock.**

Note: HP-β-CD is readily compatible with e.g. LC-MS analysis.

Plate Plan for your use:

Experiment date:……; **Test compound ID:**……; **Incubation Start/end time:** ……/…….



<u>Notes:</u>

Re-ordering for Scale-up reactions

Email enquiries@hyphadiscovery.co.uk with the PolyCYPs® isoform number with which you obtained the best yield for the product(s) of interest and the reaction volume required based on the yield observed in the screen – we recommend allowing for 50% purification loss in these calculations. Hypha will then provide a quotation for the amount of enzyme, cofactor and formulant required.

For 10 to >100 mg scale-up, Hypha offers a scale-up, purification and structural elucidation service.

Safety & Handling

The contents of this kit are not classified as hazardous substances according to GHS (US) and regulation (EC) No.1272/2008. Despite this we recommend taking precautionary measures to avoid ingestion, inhalation, skin and eye contact (Risk Phrases: R22/R36/R37/R38); always work in accordance with your local health and safety regulations. The reagent quantities used in the PolyCYPs® Screening Kit present a low safety risk when used in accordance with these instructions.

All components of the kit were prepared using reagents free from animal-derived materials and the enzyme products are filter sterilised to remove any residual microbial materials. These materials are intended for *in vitro* laboratory applications only.

Store your kit at ≤ -20°C until you are ready to use it!

Stock solutions after reconstitution:

- **PolyCYPs® Enzyme vials**: Sufficient CYP450 enzyme/ferredoxin/ferredoxin reductase for 500 µL reaction volume.
- **Cofactor vial**: 1 mL of 50 mM glucose-6-phosphate (G6P), 10 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 10 UN/ml of glucose-6-phosphate-dehydrogenase (G6PDH), 5 mM $MgCl_2$, 50 mM potassium phosphate pH 7.4.
- **Bosentan vial** (substrate for positive control): 0.5 mg of bosentan to give 20 µl of 25 mg/ml DMSO stock solution.
- **HP-β-CD vial**: Sufficient lyophilised 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) to make 500 µl at 40% (w/v).
- Final reactions: 500 µl at 0.1 mg/mL test substrate concentration.

Notes

1. **Shaking & incubation:** **Shaker speed selection depends upon shaking diameter & the type of reaction vessel . General rules: square wells are best, round are best avoided; never restrict gas exchange – CYPs need oxygen!**
    - **Eppendorf Thermomixer or similar shaker (3-5 mm diameter throw)**
        - 24-well square well block: 400 rpm in block for 0.5-2.5 ml max volume/well
        - 96-well square well block: 400 rpm in block for 50-150 µl max volume/well
        - Eppendorf tubes or 96-well round well block: **do not use due to poor aeration**
    - **Other orbital shaker (1"/2.5 m to 2"/5 cm diameter throw)**
        - 24-well square well block: 150-180 rpm in block for 0.5-2.5 ml max volume/well
        - 96-well square well block: 150-180 rpm in block for 50-150 µl max volume/well
        - 96-well round well block: **do not use – poor aeration**
    - **No shaker? – Use magnetic stirrers**.
        - Good conversions can be achieved using 0.5 ml in 16 mm Ø tubes with 2 x 5 mm stirrers at a speed of 650 rpm. Avoid larger stirrers; tests using 5 x 10 mm stirrers were dramatically worse.
    - **Temperature**
        - The recommended incubation temperature is 27°C.
        - Albeit sub-optimal, reactions can also run at room temperature (18-22°C) with a longer incubation time (e.g., 24 hours).
2. **Solvent tolerance** – Although some PolyCYPs® are more tolerant and this tolerance is substrate-specific, we recommend the following for screening:
    - **Acetonitrile, DMSO & 2-Propanol :** Do not exceed 2% v/v final reaction concentration.
    - **Ethanol and Methanol:** These have not been tested so recommend maintained below 1% v/v.
3. **Bosentan positive control conversion** – bosentan ($[M+H]^+$: 552m/z) should be converted by PolyCYP6 to its mono-oxidised compound ($[M+H]^+$: 568m/z) in excess of 90% yield at UV_{270nm}. If the conversion is less than 60% at UV_{270nm}, the reaction has not performed as expected & technical assistance should be sought from Hypha. A further oxidised product ($[M+H]^+$: 566m/z) is also often observed at up to 5% yield at UV_{270nm}.
4. **Deviations from protocol / what to avoid** – in our extensive format testing we found that using round wall multi-well blocks or Eppendorf tubes for the reaction incubations gave poor yields of conversion and so should be avoided – please use the block provided whenever possible. If this is not possible, avoid plastics not classified as low-protein binding and mix the vessels used as vigorously as possible without allowing foam to form as this can lead to protein aggregation and inactivation.
5. **Ways to improve yields** – the most influential parameters are oxygenation and substrate or product inhibition. Whilst the latter two factors are substrate (test compound) specific and can be improved with reduced dosage of test compound, the former can be addressed by referring to the shaker guide detailed above.
6. **Shelf-life** – Screening Kit components have an 18-month shelf-life. Expiry dates are based on known stability data but expected to still be active beyond this period if stored correctly due to being sealed under vacuum.
7. **Storage** – The kit is shipped at ambient temperature, but should be stored at ≤ -20C upon receipt.