PolyCYPs® Diversification Kit Protocol

Version Nov.18

Overview:

Hypha's PolyCYPs® Diversification Kit contains recombinant single-enzymes for the oxidation of organic molecules. The PolyCYPs® enzymes are selected from Hypha's talented microbial biotransformation strains. This kit is designed to permit small-scale production for purification of analogues for bioassay testing. 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 (6 x blue crimp-lid vials)**: CYP450 enzyme, ferredoxin and ferredoxin reductase. Each vial contains sufficient lyophilised recombinant enzyme complex for a reaction volume of 10 ml

  The selected PolyCYPs® provided in the Diversification Kit are: 6, 152, 166, 168, 196 & 217

- **Cofactor vials (6 x green crimp-lid vials)**: 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
- **Formulant vials (6 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 (per 10 ml reaction without HP-β-CD)

1. Remove the 24-well block from the its packaging & retain for later use; you can use the foam holder as a vial rack.

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 30 minutes

2. Dissolve test compound(s) in appropriate solvent (e.g., water, DMSO, acetonitrile or 2-propanol to make a stock solution at 25 mg/ml (for 0.1 mg/ml final substrate concentration). 260.0 µl will suffice for testing the six enzymes.

3. Add 1000 µl of cold high purity H_2O to the **cofactor** vials (1 vial per 10ml reaction), gently mix to dissolve.

4. Without mixing, add a total of 8.96 ml of cold high purity water to each of the **PolyCYPs®** enzyme vials, stand to soak for approximately 2 minutes before progressing; this reduces protein aggregation.

5. 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.

6. Dispense 40.0 µl of your test compound solution into each vial of **PolyCYPs®** enzyme solution, mix gently.

7. Dispense the contents of one **cofactor** vial (1 ml) to each **PolyCYPs®** vial.

8. Transfer the contents of each vial to the four wells per column of the 24-well plate provided (2.5 ml/well); seal the plate with the gas-permeable seal.

9. 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.

10. Terminate all reactions by adding 2.5 ml of acetonitrile to each well, or more for more apolar substrates and mix (pipetting or shaking) to ensure extraction. It is normal to occasionally observe a semi-solid aggregate in the reactions after the incubation period.

11. Collect each set of extracts per PolyCYPs® isoform into separate centrifuge tubes for processing & purification - we recommend plate wells are also rinsed with solvent for full product recovery. Before analysing each pooled reaction product, centrifuge at 1,000 x g for 10 minutes to remove insoluble materials taking the usual precautions against residual solids.

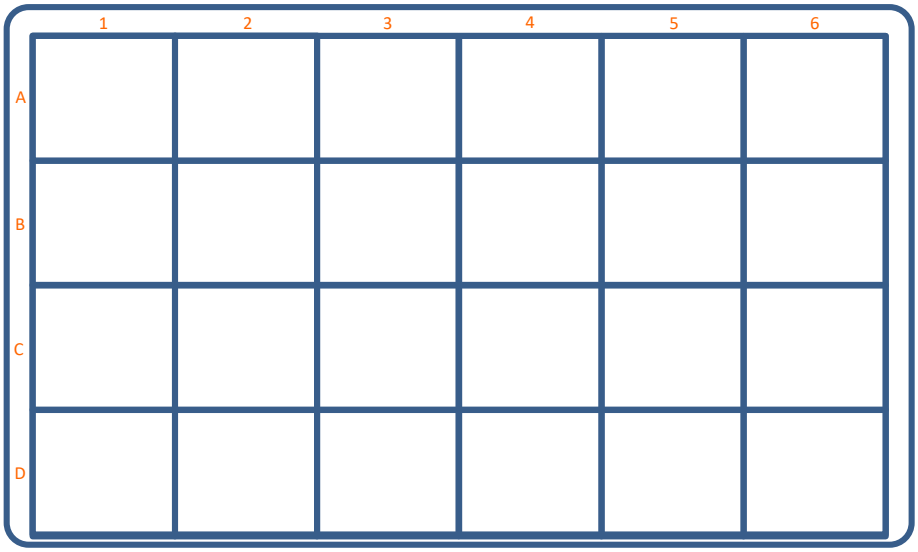
Changes to protocol <u>for substrates of solubility <0.01mg/ml</u> (10 ml reactions with HP-β-CD)

- **Replace step 2 above with:** Dissolve test compound(s) in appropriate solvent (e.g., DMSO, acetonitrile or 2-propanol to make a stock solution at 25 mg/ml. 260.0 µl will suffice for testing the six enzymes. To each **HP-β-CD** vial (one per 10ml reaction) add 40 µl of the test compound solution stock, followed by 460 µl of high purity water. Vortex and keep on the bench until use. Incompletely dissolved stocks can also be used.
- **In step 4 above change the water volume from 8.96 ml to 8.5 ml.**
- **In step 6 above change the test compound solution volume from 40 µl to 500 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

Email enquiries@hyphadiscovery.co.uk with the PolyCYPs® isoform number for which you require additional reaction volume – we recommend allowing for at least 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:

- **Each PolyCYPs® vial**: Sufficient CYP450 enzyme/ferredoxin/ferredoxin reductase for 10 ml reactions per vial
- **Each 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
- **Each HP-β-CD vial**: Sufficient lyophilised 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) to make 500 µl at 40% (w/v)
- Reactions using one of each of the above provides 10 ml at e.g., 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. **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.
4. **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.
5. **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.
6. **Storage** – The kit is shipped at ambient temperature, but should be stored at ≤ -20˚C upon receipt.
7. **Further scale-up** – When the volume required is ≥500 ml – 1.5 L we have stock ready for reactions using freshly prepared enzyme available on a service basis. For greater reaction volumes, Hypha has both the originating wild-type microbial strain from which the enzymes originated, as well as genetically engineered *Streptomyces* strains constitutively expressing each of the PolyCYPs® isoforms for multi-litre production.