PolyCYPs® Diversification Kit (8 CYPs) Protocol

Overview

This kit contains 8 PolyCYPs recombinant enzymes proficient at oxidizing a wide range of compounds, in addition to PolyCYP 6 which is used for the control reaction with bosentan. PolyCYPs® CYP enzymes are cloned from Hypha's microbial biotransformation strains and are capable of hydroxylating a wide range of substrate compounds with aliphatic and aromatic C-H systems to form alkyl-hydroxyls, phenols and epoxides. De-alkylation of *N*- and *O*-alkyl moieties is also observed. The PolyCYPs® CYP enzymes are the subjects of multiple patent applications; use of these materials is limited to the intended purpose described above.

What's in the box?

- **Enzyme vials (blue crimp-lid vials)**: Lyophilised enzyme preparations with buffer contained therein. The enzymes provided in this kit are shown on the plate plan (page 2).
- **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 8.
- **Bosentan (1 x silver crimp-lid vial)**: Substrate for the positive control reaction with PolyCYP 6.
- **Formulant (1 x red crimp-lid vial)**: 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) NB: only use the formulant 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 (opaque appearance)

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

1. Unpack all the kit contents & check against the list above; discard the clear block seal & remove all vials from the 24-well block (keep the block for later). Open all the vials (pliers recommended for easier metal crimp removal). Note the vials are vacuum-sealed – release vacuum slowly.

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. Without mixing, add a total of 448 µl of cold high purity water to each of the PolyCYPs enzyme vials, stand for approximately 5 minutes before progressing.

5. After the soaking time, **gently** agitate the PolyCYPs enzyme vials using a pipette until a clear 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. Add 1050 µl of cold high purity H_2O to the cofactor vial, gently mix to dissolve.

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 PolyCYP 6 enzyme vial provided, mix gently.

9. Dispense 50 µl of the cofactors solution to all PolyCYPs vials, including the bosentan + PolyCYPs 6 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 (opaque seal) provided in the packaging sleeve (not the clear seal that held the vials in the block).

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 and be wary of evaporation at higher temperatures.

12. Terminate all reactions by adding at least 500 µl of acetonitrile to each well, ideally 1000-1500 µl as this aids protein precipitation and can help with dissolution of more apolar substrates. Mix thoroughly by pipetting or shaking. It is normal to occasionally observe a semi-solid aggregate in some reactions after the incubation period.

13. Allow the samples to stand at room temperature for at least 30-60mins to encourage protein aggregation/precipitation.

14. Centrifuge the 24-well block with reaction extracts *in-situ* or transfer to centrifuge tubes. Centrifuge the samples using a microfuge at maximum speed for tubes, or in a bench-top centrifuge at 4,000 x g for 20 minutes for plates.

15. Transfer supernatants to vials/plates for analysis. Samples should be analysed as soon as possible after centrifugation. Samples left to stand for an extended period could precipitate so should be recentrifuged before analysis.

Changes to protocol <u>for substrates of solubility <0.01mg/ml</u> (500 µl reactions with HP-β-CD) Page 2

- **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 42 µl of the test compound solution stock to the **HP-β-CD** vial followed by 483 µl of high purity water. Vortex and keep on the bench until use.
- **In step 4 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 contents:

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

	1	2	3	4	5	6
A	CYP152	CYP350				CYP006 Control
B	CYP166	CYP353				Bosentan control
C	CYP168	CYP359				Co-factor
D	CYP333	CYP484				Formulant

<u>Notes:</u>

Re-ordering for Scale-up reactions

Email enquiries@hyphadiscovery.com with the PolyCYPs isoform number(s) 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. However, 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!

Solution compositions after reconstitution:

- **PolyCYPs® Enzyme vials**: Sufficient enzyme and buffer components for 500 µl reaction volume.
- **Cofactor vial**: 1.05 ml of 50 mM glucose-6-phosphate (G6P), 10 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 10 UN/ml of glucose-6-phosphate-dehydrogenase (G6PDH), 4.76 mM $MgCl_2$, 95.2 mM potassium phosphate pH 8.
- **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 525 µl at 38% (w/v).
- **Final reactions**: 500 µl at 0.1 mg/mL test substrate concentration if performed as instructed.

Notes

1. Incubation conditions

The optimum shaker speed depends upon shaker diameter and the type of reaction vessel. Use only square-well block formats with the gas permeable seal provided. Do not use Eppendorf tubes or round-well blocks due to poor aeration. Apply volume limits - 96-square well: 50-150 µl max./well; 24-square well: 0.5-2.5ml/well.

Eppendorf Thermomixer or similar shaker (1.5-5 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.

Other orbital shakers (e.g., 2 cm to 5 cm diameter throw)

- Use the handy calculator on our website e.g. 150 rpm for a 5 cm orbit shaker.

Never restrict gas exchange – the reactions need oxygen

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 gave very poor results.

2. Temperature - the recommended incubation temperature is 27°C. If you need to run at room temperature (18-22°C), use a longer incubation (e.g. 24 hours). Avoid higher temperatures as these lead to excessive evaporation.

3. Solvent tolerance –we recommend the following solvents and maximum concentrations:

- **Acetonitrile, DMSO & 2-Propanol:** Do not exceed 2% v/v final reaction solvent concentration. Ethanol and methanol have not been tested so not recommended.

4. Bosentan positive control conversion – bosentan ($[M+H]^+$: 552m/z) should be converted by PolyCYP6 to its oxidised products ($[M+H]^+$: 568m/z, 566m/z & 582m/z) in excess of 90% substrate conversion at UV_{270nm}. If the conversion is less than 80% at UV_{270nm}, the reaction has performed sub-optimally – seek advice from Hypha.

5. Deviations from protocol / what to avoid – using round wall multi-well blocks or Eppendorf tubes for the incubations give very poor conversion yields and should be avoided –use the block provided whenever possible. If this is not possible, use low-protein binding plastics and mix the vessels used as vigorously as possible without allowing foam to form as this can lead to protein aggregation and inactivation. Phosphate buffer/MeCN mixtures can form biphasic systems when cooled, compromising analyses. This can be resolved by ensuring samples are mixed once returned to room temperature. Eppendorf tubes can be used for post-extraction centrifugation.

6. Ways to improve yields – the most influential parameters are oxygenation as well as substrate and/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. Shaker speeds should be as high as possible without forming a persistent foam or risk to the block detaching.

7. Shelf-life – Each vial in the kit has a unique expiry date and the kit 'use-by' date is based on the earliest expiring component. Hypha will not supply kits with less than 3 months' remaining time before the expiry date.

8. Storage – The materials are stable at a temperature up to 27˚C for 10 days as long as the vials remain sealed, but should be stored at ≤ -20˚C upon receipt. Once vials are opened the contents must be used straightaway as exposure to air will reduce the enzyme systems effectiveness over a few days.