PolyCYPs®+ Screening Kit Protocol

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Overview:

Hypha's PolyCYPs®+ screening kit contains 20 recombinant enzymes for the oxidation of organic molecules. The PolyCYPs® CYP enzymes are cloned from Hypha's talented 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®+ kit also contains recombinant human aldehyde oxidase (AOX1) and flavin-containing monooxygenase (FMO3) enzymes for heteroaromatic oxidation and *N*- or *S*-oxidation. 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. Two copies of PolyCYPs® 6 are provided, one extra for the positive control reaction using the bosentan substrate provided. The enzymes provided in the 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 7.4. This NADPH regeneration system is needed by the CYP & FMO3 enzymes for activity, but not for AOX activity.
- **Bosentan (1 x silver crimp-lid vial)**: Substrate for positive control with the extra PolyCYPs® 6 vial.
- **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 (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. 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); **reduce the stock strength for lower molecular mass compounds to ensure the screening concentration is ~200-300µM or below.**

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

4. Add 1050 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 FMO and **bosentan+PolyCYPs® 6** positive control reaction. The AO reaction works with or without these cofactors, so add 50 µl water instead.

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 500 µl of cold acetonitrile to each well (1000-1500 µl can be used to ensure dissolution of more apolar substrates and further aid protein precipitation) & mix thoroughly to avoid a biphasic sample (pipetting or shaking). It is normal to occasionally observe a semi-solid aggregate in some reactions after the incubation period. Addition of methanol can be used to eliminate any biphasic sample forming on storage.

13. Allow the samples to stand for 30-60mins, ideally in a fridge 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 greater than 1,000 x g for at least 10 minutes to remove insoluble materials to clarify extracts prior to analysis.

15. Transfer supernatants to appropriate vials/plates for analysis. Take usual precautions against residual solids.

PolyCYPs® Screening Kit Protocol – Extended Kit

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 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 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:** ……/…….

	1	2	3	4	5	6
A	CYP006	CYP194	CYP333	CYP353	CYP486	
B	CYP152	CYP196	CYP334	CYP359	CYP488	
C	CYP166	CYP217	CYP349	CYP483	FMO3	
D	CYP168	CYP235	CYP350	CYP484	AOX1	CYP006 Control

<u>Notes:</u>

(CYPs and FMO need the cofactor; AOX does not)

Re-ordering for Scale-up reactions

Email enquiries@hyphadiscovery.co.uk 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!

Stock solutions after reconstitution:

- **PolyCYPs® Enzyme vials**: Sufficient enzyme and buffer components for 500 µL reaction volume.
- **Cofactor vial**: 1.05 mL of 47.6 mM glucose-6-phosphate (G6P), 9.5 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 9.5 UN/ml of glucose-6-phosphate-dehydrogenase (G6PDH), 4.76 mM $MgCl_2$, 47.6 mM potassium phosphate pH 7.4. CYPs & FMO require this cofactor NADPH regeneration system, AOX does not.
- **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.

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.

Never restrict gas exchange – the reactions need oxygen

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 yields 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 avoided by use of MeOH:MeCN 1:1 for extraction, subject to any MeOH incompatibility. 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. Only accelerated stability tests have been performed on the FMO and AO vials, therefore these are complimentary additions to the kit and stability testing is ongoing – users will be alerted and new vials provided if any issues are found.

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.