PolyCYPs® Scale-up Kit Protocol

Version Nov.19

Overview:

Hypha's PolyCYPs® Scale-up Kits contain one or more vials of a specified enzyme isoform for resupply in larger amounts, generally arising as a result of testing compounds in the PolyCYPs+ screening kit. Each scale-up vial is supplied with a co-factor vial (where applicable), formulant & incubation vessel with seal. Each vial provides 10ml when reconstituted which, as a guide is typically incubated with 1 mg of parent compound. PolyCYPs+ enzymes include PolyCYPs® CYP enzymes cloned from Hypha's talented microbial biotransformation strains, as well as non-CYP recombinant human aldehyde oxidase (AOX1) and flavin-containing monooxygenase (FMO3) enzymes. The PolyCYPs® CYPs 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?

- **PolyCYPs® Enzyme vials (blue crimp-lid vials)**: Lyophilised enzyme preparations with buffer contained therein. Each vial contains sufficient lyophilised recombinant enzyme complex for a reaction volume of 10 ml
- **Cofactor vials (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. This NADPH regeneration system is needed by the CYP & FMO3 enzymes for activity, but not for AOX activity
- **Formulant vials (red crimp-lid vials)**: 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 (per 10 ml reaction without HP-β-CD)

1. Unpack all the kit contents & check against the contents list above; you can use the foam holder as a vial rack. It is recommended to perform the reaction using one scale-up vial for an initial dose ranging experiment before progressing with the remaining vials. Dose range should be both higher & lower to that used in the screen.

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. Open all the vials when ready to start. Note: The vials are vacuum-sealed – release vacuum slowly

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). 40.0 µl is needed per 10 ml reaction volume.

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 (or 1ml H_2O for AO scale-up)

8. Transfer the contents of the vials to the wells of the 24-well plate provided (2.5 ml/well); seal the plate with the gas-permeable seal (opaque seal) provided in the plate packaging.

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 and be wary of evaporation at higher temperatures.

10. Terminate all reactions by adding solvent, e.g., 2.5 ml of acetonitrile to each well, or more for more apolar substrates & mix thoroughly (pipetting or shaking) to ensure extraction. It is normal to occasionally observe a semi-solid aggregate in the reactions after the incubation period. Addition of methanol can be used to eliminate any biphasic sample forming on storage.

11. Allow the samples to stand for 30-60mins, ideally in a fridge to encourage protein aggregation/precipitation.

12. Collect the extract into centrifuge tubes for processing & purification - we recommend plate wells are also rinsed with solvent for full product recovery. Before analysing, centrifuge at 1,000 x g for at least 10 minutes to remove insoluble materials taking the usual precautions against residual solids.

PolyCYPs®+ Scale-up Kit Protocol

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. Each 10ml reaction requires 40 µl of this stock. 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:** ……/…….

	1	2	3	4	5	6
A						
B						
C						
D						

<u>Notes:</u>

Re-ordering

Email enquiries@hyphadiscovery.co.uk with the PolyCYPs® isoform number for which you require additional enzyme material – we recommend allowing for at least 50% purification loss in these calculations, plus a vial for optimisations. 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 enzyme and buffer components 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)
- One of each of the above provides 10 ml at 0.1 mg/mL test substrate concentration

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 as specified below.

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.

Never restrict gas exchange – the reactions need oxygen

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 in 16 mm Ø tubes 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. 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.

5. 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.

6. 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. Only limited stability tests have been performed on the FMO and AO vials, which were included free in the screening kit. Users will be alerted and new vials provided if any issues are found.

7. Storage – The materials are stable at a temperature up to 27˚C for 10 days as long as the vials remain vacuum-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.

8. Further scale-up – When the volume of reaction that is required is greater than 500 ml – 2 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.