PolyCYPs® Scale-up Kit Protocol

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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 as well as non-CYP recombinant human aldehyde oxidase (AOX1) and flavin-containing monooxygenase (FMO3) enzymes. PolyCYPs® 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 8. 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 (pliers recommended for metal crimp removal). 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. 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 a minimum of 5-10 minutes, ideally in an ice bath, before progressing.

4. 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.

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

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 aware of evaporation at higher temperatures.

10. Terminate all reactions by adding an equal volume of the chosen organic solvent to each well e.g. 2.5ml acetonitrile, or more for more apolar substrates, & mix thoroughly (pipetting or shaking) to ensure extraction. For samples for LC-MS analysis, a solvent:sample ratio of up to 3:1 is recommended to improve protein precipitation. It is normal to occasionally observe a semi-solid aggregate in the reactions after the incubation period.

11. Allow the samples to stand at room temp for 30-60mins to encourage protein precipitation before centrifugation.

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, samples should be centrifuged to pellet protein, either in tubes using a microfuge at maximum speed for 10 mins or in plates in a bench-top centrifuge at 4,000xg for 20 minutes.

13. Transfer supernatents to vials for analysis. Samples should be analysed as soon as possible after centrifugation. Any samples left to stand may further precipitate so should be re-centrifuged prior to analysis.

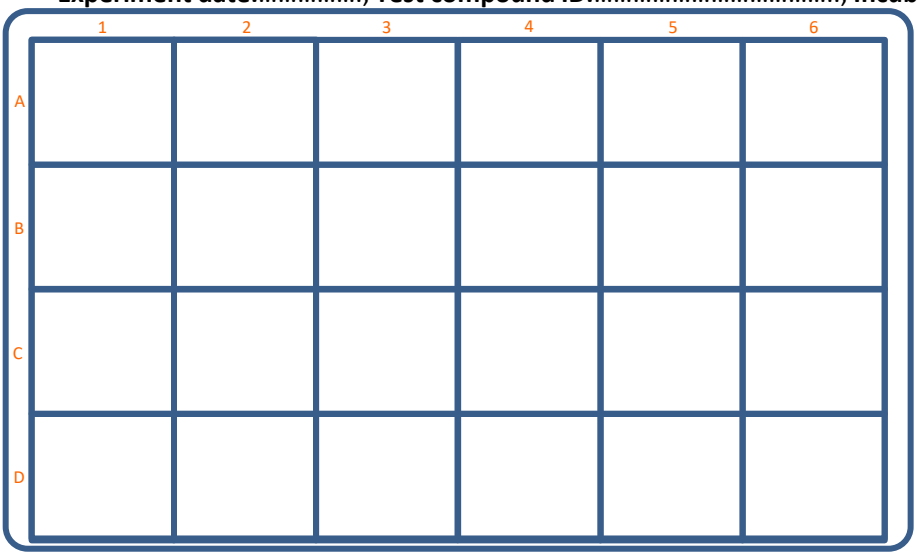
Changes to protocol <u>for substrates of solubility <0.01mg/ml</u> (10 ml reactions with HP-β-CD)

- **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 3 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.com 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

Please refer to the Safety Data Sheet According to Regulation (EC) No. 1907/2006, as amended by UK SI 2019/758, available on Hypha's website at https://www.hyphadiscovery.com/polycyps-kit-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:

- **Each PolyCYPs® vial**: Sufficient enzyme and buffer components for 10 ml reactions per vial
- **Each Cofactor vial**: 1 ml of 52.5 mM glucose-6-phosphate (G6P), 10.5 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 10.5 UN/ml of glucose-6-phosphate-dehydrogenase (G6PDH), 5 mM $MgCl_2$, 100 mM potassium phosphate pH 8.
- **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 of reaction 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 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.

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.

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 recombinant Streptomyces strains constitutively expressing the PolyCYPs enzymes, as well as the originating microbial strains for wild-type isoforms.