PolyUGTs® Scale-up Kit Protocol

Version 1 (12/03/2025)

Overview:

Hypha's PolyUGTs® Scale-up Kit contains vials of the selected glucuronosyltransferase enzyme isoform for preliminary metabolite production, generally after testing compounds in the PolyUGTs® Screening Kit. Each scale-up vial is supplied with UDPGA cofactor, formulant, pH reduction reagent, and incubation vessel with seal. Each vial provides 10 mL when reconstituted which, as a guide is typically incubated with 1 mg of parent compound. PolyUGT® enzymes are capable of glucuronidating a wide range of substrate compounds with aliphatic and aromatic hydroxyls, nitrogen-containing systems, or acyl moieties to form *O*-, *N*- *or* acyl-glucuronides, respectively. Use of these materials is limited to the intended purpose described above.

What's in the box?

- **PolyUGT® Enzyme vials (gold crimp-lid vials)**: Lyophilised enzyme preparations with buffer contained therein. Each scale-up vial contains sufficient lyophilised enzyme for a reaction volume of 10 mL per vial.
- **UDPGA cofactor vials (black crimp-lid vial)**: Uridine diphosphate glucuronic acid (UDPGA) is needed by the enzyme for glucuronidation activity.
- **Formulant vials (red crimp-lid vials)**: 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD). NB: only use for test compounds with aqueous solubility <0.01 mg/mL
- **pH reduction vial: (pink crimp-lid vial)**: Potassium phosphate monobasic NB: only use when targeting production of acyl-glucuronides to minimise acyl migration. Testing with and without pH reduction can reveal migration susceptibility.
- **24-square well polypropylene block**: To be used for incubation of dose-escalation experiments at 0.5 mL reaction per well, or for scale-up reactions if required should glass flasks, beakers, or centrifuge tubes not be readily available (see Note 1, p3).
- **Self-adhesive foil plate seal**: To be used to seal the plate during incubation.

Step by step protocol (per 10 mL reaction without HP-β-CD)

1. Unpack all the kit contents and 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 and lower to that used in the screen (typically 0.1 mg/mL), e.g., 0.05-0.3 mg/mL. **During the following steps it is recommended to use an ice bath for the reconstituted enzyme and 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 sufficient stock solution for 40 µL per 10 ml reaction volume (e.g., for 0.1 mg/mL test compound concentration in reaction prepare a stock at 25 mg/mL).

3. Without mixing, add a total of 8.96 mL of cold high purity water to each of the **PolyUGTs® 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 **PolyUGTs® 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. For test compounds shown from screening to have improved yields using reduced pH conditions, such as some acyl-glucuronides, a pH reduction vial (1 per 10 mL reaction) is provided to decrease the pH of reaction from 7.4 to 6.5. If not tested at the screening stage, use of the reduced pH can be assessed as part of further optimisation if needed; however, if not known or tested do not use pH reduction.

5a. Add 1000 µL of cold high purity water to the **pH reduction vial**, vortex to dissolve.

5b. Dispense the contents of one **pH reduction vial** (1 mL) to each **PolyUGTs® vial** (or separately for optimisation test solutions).

6. Unless performing a dose-escalation experiment, dispense 40 µL of your test compound solution into each vial of **PolyUGTs® enzyme solution**, mix gently. For dose-escalation experiments, add 2 µL of compound at selected concentrations per well, then aliquot 0.448 mL of enzyme material per well.

7. Add 1000 µL of cold high purity water to each **UDPGA cofactor vial** (1 vial per 10 mL reaction), gently mix to dissolve.

8. Dispense the contents of one **UDPGA cofactor vial** (1 mL) to each **PolyUGTs® vial**. For dose-escalation experiments, add 50 µL per well.

9. Transfer the contents of the vials to the incubation vessel (**see Note 1, p3**) and seal to avoid any additional gas exchange.

10. Incubate (16-20hrs) with slow/occasional agitation (**see Note 1, p3**), ideally at ~27˚C. Allow longer incubation for lower temperatures.

11. Terminate all reactions by adding an equal volume of the chosen organic solvent to reactions e.g., acetonitrile, and mix thoroughly (pipetting, vortex or shaking) to ensure extraction and avoid phase separation sometimes seen with ACN-buffer mixtures.

12. Allow the samples to stand at room temp for 30-60 minutes to encourage protein aggregation/precipitation before centrifugation.

13. Collect the extract into centrifuge tubes for processing and purification – we recommend reaction vessels 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 minutes or in plates in a bench-top centrifuge at 4,000xg for 20 minutes.

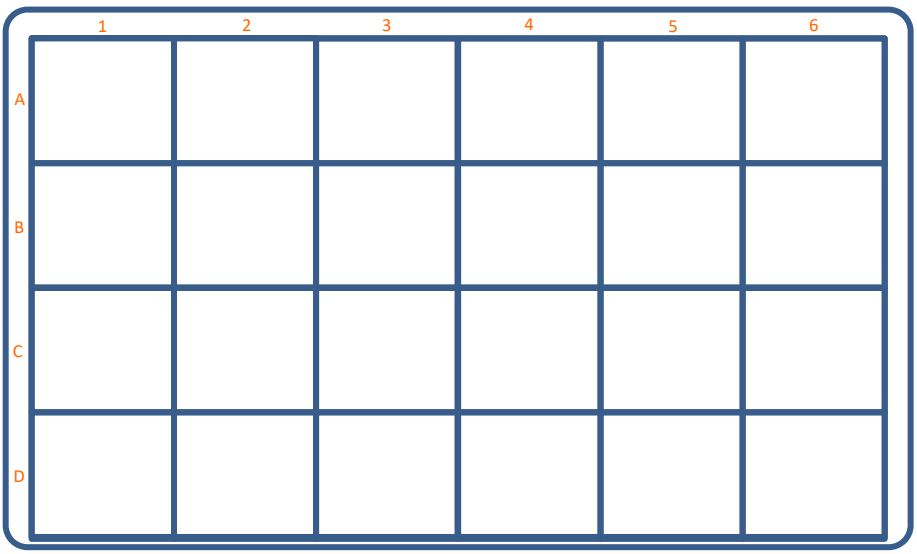
14. Transfer supernatants to vials for analysis. Samples should be analysed as soon as possible after centrifugation. Any samples left to stand may further precipitate so should be re-centrifuged prior to analysis; pool as/if needed for onward purification processing.

Changes to protocol <u>for substrates of solubility <0.01 mg/mL</u> (10 mL 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 to make sufficient stock solution for 40 µL per 10 mL reaction volume (e.g., for 0.1 mg/mL test compound concentration in reaction prepare a stock at 25 mg/mL). To each **HP-β-CD** vial (one per 10 mL reaction) add 40 µL of the test compound solution stock, followed by 460 µL of high purity water to create the formulated compound stock. 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 (or 2 µL to 25 µL, for dose-escalation experiments).
- **Note:** HP-β-CD is readily compatible with e.g., LC-MS analysis.

Plate Plan for dose escalation tests if required; 0.5 mL reaction/well:

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



<u>Notes:</u>

Re-ordering

Email enquiries@hyphadiscovery.com with the PolyUGTs® 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 specific Safety Data Sheet relevant to your region, available on Hypha's website at:
https://www.hyphadiscovery.com/polyugts-kit-instructions/

Always work in accordance with your local health and safety regulations 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 PolyUGTs® vial**: Sufficient enzyme and buffer components for 10 mL reaction per vial
- **Each UDPGA Cofactor vial**: 1 mL of 50 mM Uridine diphosphate glucuronic acid trisodium salt in DI water. UGT enzymes require this cofactor for glucuronidation
- **Each HP-β-CD vial**: 500 µL of formulated stock at 40% (w/v) 2-hydroxypropyl-β-cyclodextrin (HP-β-CD).
- **pH reduction vial**: 1 mL of 1.3 M Potassium phosphate monobasic in water.
- **Final reactions:** 10 mL at 0.1 mg/mL test substrate concentration if performed as instructed. If pH reduction vial is used, all reaction components will be diluted by approximately 10% (i.e., 0.09 mg/mL test substrate concentration).

Notes

1. Incubation conditions

Reaction **only requires slow and gentle agitation to mix** the reaction components; higher speeds reduce PolyUGT activity.

For best results pool vials into sealed flasks and mix gently on orbital shakers with a 1 cm to 5 cm Ø throw

- e.g., 30-60 mL in 250 mL flask at 75 rpm for a 5 cm orbit shaker, or 10-20 mL per 50 mL centrifuge tube at low rpm

Equivalent conversions can also be achieved using magnetic stirrers

- e.g., up to 60 mL in 250 mL flask with magnetic stirrers at very slow speeds without surface agitation, e.g., 50-80 rpm.

Static incubation (if the user does not have access to any of the above equipment/consumables)

- Static incubation can reduce scale-up yields by 10-20% compared to screening conditions
- 24-well block (provided): mix reactions gently, and then incubate standing/static with occasional manual mixing.
- Product vials in situ: mix reactions gently, and then incubate standing/static with occasional manual mixing.

NB: DO NOT USE small orbit shakers <1 cm Ø throw, e.g., Eppendorf Thermomixer, Titramax 101 or similar (1.5-5 mm Ø throw) as these can reduce scale-up yields by 20-40% compared to screening conditions

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 reduce activity.

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

- **DMSO, Methanol, 2-Propanol, and Acetonitrile:** Do not exceed 10% DMSO, 5% MeOH, 2% IPA, and 2% ACN (v/v final reaction solvent concentration). Ethanol has not been tested so not recommended.

4. Deviations from protocol / what to avoid – Ensure vessels are sealed and mixed gently – overmixing causes unneeded oxygenation which can lead to protein inactivation and poor conversion yields. Please contact us if you require advice on the equipment you have available to confirm your intended scale-up protocol.

5. Ways to improve yields – the most influential parameters are substrate and/or product inhibition, which can be improved with titrating dosage of test compound and or UDPGA cofactor. Shaker speeds should be set to be just sufficient to ensure gentle mixing without surface disturbance.

6. Extraction – Phosphate buffer/ACN mixtures can form biphasic systems when cooled, compromising analyses; this can be resolved by ensuring samples are mixed once returned to room temperature or using an additional solvent like methanol subject to any incompatibility. Eppendorf tubes can be used for post-extraction centrifugation.

7. Shelf-life – Each vial in the kit has a unique expiry date upon which the kit 'use-by' date is based using 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 vacuum-sealed, but should be stored at ≤ -20˚C upon receipt. Once vials are opened the contents should be used straightaway as exposure to air can reduce the enzyme systems effectiveness over a few days.

9. Further scale-up – When the volume of reaction that is required is greater than that realistically achievable by scale-up vials, we have stock ready for reactions using freshly prepared enzyme available on a service basis. For greater metabolite material amounts, Hypha has recombinant *Streptomyces* strains constitutively expressing the PolyUGTs enzymes, originating microbial strains for wild-type isoforms, as well as other proprietary synthesis approaches.