Overview

Hypha's glucuronidation screening kit contains 11 different recombinant enzymes for the glucuronidation of organic molecules. The glucuronosyltransferase (UGT) enzymes are cloned from some of Hypha's talented microbial biotransformation strains and are capable of glucuronidating a wide range of substrate compounds with aliphatic and aromatic hydroxyl, nitrogen-containing systems, or acyl moieties to form *O-*, *N- or* acyl-glucuronides, respectively. Use of these materials should be 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 vial has enough material to run the reactions at 500 µL or in duplicate 250 µL reactions as shown in the plate plan.
- **UDPGA cofactor vial (Black crimp-lid vial)**: Uridine diphosphate glucuronic acid (UDPGA) is needed by the enzyme for glucuronidation activity.
- **4-Methylumbelliferone (4-MU) vial (1 x silver crimp-lid vial)**: Substrate for positive control with the extra UGT216 vial.
- **Formulant vial (1 x red crimp-lid vial)**: 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) NB: only use for test compounds with aqueous solubility <0.01 mg/mL.
- **pH reduction vial: (1x 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 and may increase yields of the target acyl glucuronide when run at pH 6.5.
- **24-square well polypropylene plate**: To be used for incubation once the reactions are prepared.
- **Self-adhesive foil plate seal**: To be used to seal the plate during incubation.

Plate Plan for your use:

Experiment date_____; Test compound ID:_____; Incubation Start/end time: _____/_____]

	1	2	3	4	5	6
A	UGT177	UGT194	UGT213	UGT177	UGT194	UGT213
B	UGT178	UGT203	UGT214	UGT178	UGT203	UGT214
C	UGT179	UGT204	UGT216	UGT179	UGT204	UGT216
D	UGT193	UGT207	UGT216 4-MU Control	UGT193	UGT207	UGT216 4-MU Control

Notes:

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!

Step by step protocol (Reactions without HP-β-CD)

1. Unpack all the kit contents and check against the list above; discard the clear block seal and remove all vials from the 24-well block (keep the block for later). Open all the vials. Pliers are 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 and 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 of stock solution (e.g., for 0.1 mg/mL test compound concentration in reaction prepare a stock at 25 mg/mL).

3. Add 20 µL of DMSO to the **4-methylumbelliferone (4-MU) vial**, vortex and keep at room temperature before use.

4. Without mixing, add a total of 448 µL of cold high purity water to each of the **PolyUGT® enzyme vials**, stand for approximately 5 minutes before progressing.

5. After the soaking time, **gently** agitate the **PolyUGT® 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. There is enough PolyUGT® enzyme material per isoform to run one or two reactions (e.g., for either duplicate reactions per isoform, or testing a compound at two different compound concentrations, or comparing with and without pH reduction)**:**

6a. Single 500 µL reactions: Setting the extra UGT216 enzyme vial aside (control), dispense 2 µL of the test compound solution into each **PolyUGT® vial**, mix gently. Dispense 2 µL of the **4-MU** solution to the **extra UGT216 enzyme vial** provided, mix gently. Transfer the entire contents of each **PolyUGT®** vial into the specified individual well (one well per reaction per isoform). **Proceed to Step 7 (if required) or Step 8.**

6b. Two 250 µL reactions: dispense 1 µL of the test compound solution into each test well. Dispense 1 µL of the **4-MU** solution to the control wells; then dispense two aliquots of the **PolyUGT® enzyme solutions** by transferring 224 µL into the specified individual wells as per the example plate plan provided. **Proceed to Step 7 (if required) or Step 8.**

7. For screening compounds containing carboxylic acids for acyl-glucuronide formation, a pH reduction vial is provided to decrease the pH of reaction from 7.4 to 6.5, to minimise acyl-glucuronide migration. If test compound does not contain any carboxylic acid groups or acyl-glucuronides are not the target metabolite, do not add pH reduction solution as yields of non-acyl-glucuronide metabolites are affected.

7a. Add 1000 µL of cold high purity water to the **pH reduction vial**, vortex to dissolve.

7b. 500 µL reactions: Add 50 µL of pH reduction solution to each reaction.

7c. 250 µL reactions: Add 25 µL of pH reduction solution to each reaction; for the non-pH reduced control set, add 25 µL water.

8. Prepare **UDPGA cofactor:** Add 1000 µl of cold high purity water to the **UDPGA cofactor vial**, gently mix to dissolve.

8a. 500 µL reactions: Add 50 µL of **UDPGA cofactor** solution to each reaction, including the **4-MU/UGT216** positive control reaction.

8b. 250 µL reactions: Add 25 µL of **UDPGA cofactor** solution to each reaction, including the **4-MU/UGT216** positive control reaction.

9. Seal the plate with the self-adhesive foil seal provided in the packaging sleeve (not the clear seal that held the vials in the block).

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

11. Terminate all reactions by adding at least an equal reaction volume of acetonitrile to each well. Mix thoroughly (pipetting or shaking).

12. Allow the samples to stand at room temperature for at least 30-60 mins, or longer if possible to encourage protein aggregation/precipitation before processing for analysis.

13. Centrifuge the 24-well block with reaction extracts *in-situ* or transfer to centrifuge tubes. Centrifuge the samples either using a microfuge at max speed for 10 minutes (for tubes), or in a bench-top centrifuge at 4,000xg for 20 minutes for plates, to remove insoluble materials and clarify extracts prior to analysis.

14. Transfer supernatants to appropriate vials/plates for analysis as soon as possible. Samples left to stand for extended periods of time could further precipitate so should be centrifuged again prior to analysis.

Changes to protocol for substrates of solubility <0.01 mg/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 **minimum of 50 µL stock solution** (e.g., for 0.1 mg/mL test compound concentration in reaction prepare a stock at 25 mg/mL). Add 42 µL of this compound solution to the **HP-β-CD** vial, followed by 483 µL of high purity water to create the formulated compound stock.
- **In step 4** above change the water volume from 448 µL to 425 µL.
- **In steps 6a and 6b** above for 500 µL and 250 µL reaction volumes change the test compound solution volumes to 25 µL and to 12.5 µL of formulated compound stock, respectively. For **6b**. **250 µL reactions**, dispense two aliquots of enzyme by transferring 212.5 µL.
- **Note:** HP-β-CD is readily compatible with e.g., LC-MS analysis.

Solution compositions after reconstitution:

- **PolyUGT® enzyme vial**: Sufficient enzyme and buffer components for 500 µL (single) or 250 µL (duplicate) reaction volume.
- **UDPGA cofactor vial**: 1 mL of 50 mM Uridine diphosphate glucuronic acid trisodium salt in water. UGT enzymes require this cofactor for glucuronidation.
- **4-Methylumbelliferone vial (4-MU)** (substrate for positive control): 20 µL of 25 mg/mL of 4-methylumbelliferone in DMSO.
- **HP-β-CD vial**: 525 µL of formulated stock at 38% (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**: 500 µL 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 use orbital shakers with a 1 cm to 5 cm Ø throw

- Use the handy PolyUGT shaker speed calculator on our website e.g., 100 rpm for a 5 cm orbit shaker.

Other small orbital shakers <1 cm Ø throw, e.g., Eppendorf Thermomixer, Titramax 101 or similar (1.5-5 mm Ø throw)

- **24-well block:** use minimum rpm at 0.25-0.5 mL/well. **96-well block:** use minimum rpm at 50-150 µL/well.
- **NB: Scale-up kit reactions require different conditions**

2. Temperature – the recommended incubation temperature is 27°C for 16-20 hours; higher temperatures 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.

4. 4-methylumbelliferone positive control conversion – 4-methylumbelliferone ($[M+H]^-$: 175m/z) should be converted by UGT216 to its glucuronidated product ($[M+H]^-$: 351m/z) in excess of 90% substrate conversion at UV_{320nm}. If the conversion is less than 90% at UV_{320nm}, the reaction has performed sub-optimally – seek advice from Hypha.

5. Acyl glucuronide migration – A pH reduction vial is supplied with the kit to decrease the reaction pH from 7.4 to 6.5, used to reduce acyl migration which can increase yields; at pH <6.5 the enzyme becomes inactive. Other steps to reduce acyl migration include shortening incubation times (i.e., testing/sampling at 1, 2 or 3 hours) and addition of formic acid (not supplied) after incubation is complete to a final concentration of 2% before addition of your extracting solvent.

If test compound does not contain any carboxylic acid or acyl-glucuronides are not the target metabolite, do not add the pH reduction solution as it will compromise the activity of the enzyme for some *N*- and *O*- glucuronidation reactions.

NB: You should still obtain an excess of 90% substrate conversion with the 4-methyl-umbelliferone positive control reaction with the pH reduction solution added.

6. Deviations from protocol / what to avoid – use the block provided whenever possible. If this is not possible, use new low-protein binding plasticware e.g., other plates or centrifuge tubes. Ensure vessels are sealed and mix gently – overmixing causes unneeded oxygenation which can lead to protein inactivation and poor conversion yields.

7. 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, foaming or risk to the block detaching.

8. 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.

9. 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.

10. Storage – The materials should be stored at ≤ -20˚C upon receipt. Once vials are opened the contents should be used immediately for best performance.

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