pNPG assay

Preparation:

1. Prepare pNPG stock solution in water at 10 mM

2. Prepare enzyme working solution (1 m/ml) from the stock solution (dilute the crude fecal enzymes with KPI to afford a working solution at 1 mg/mL)

3. Prepare the quenching solution (5% formic acid in acetonitrile).

Steps:

1. For each well, add KPI buffer (different pH, different magnesium concentration) 180 -X uL.

2. Add enzyme working solutions (different enzymes, mice, rats vs humans; fresh, 1 day vs 7 day, sonication vs suspension…) X µL (This volume is calculated from the final enzyme concentration)

3. Add pNPG working solution

4. Incubate ?? min at 37 degree

5. Add 20 uL of quenching solution

6. Read the plate to obtain the OD value.

KPI	Enzyme working solution (1 mg/mL)	pNPG (10 mM)	**Total**
180-X µL	X µL	20 µL	**200 µL**

X is calculated from the final enzyme concentration. For example, if your final enzyme concentration is 50 ug/mL, X should be 1 mg/mL/0.05 mg/mL= 20 fold dilution, which means take 10 uL of enzyme working solution into the system (total is 200 uL).

Glucuronides assay

Preparation:

1. Prepare glucuronide stock solution in water at 1 mM.

2. Prepare enzyme working solution (1 m/ml) from the stock solution (dilute the crude fecal enzymes with KPI to afford a working solution at 1 mg/mL)

3. Prepare the quenching solution (5% formic acid in acetonitrile).

4. Label sample and reaction tubes (1.7 mL, n=3)

5. Chilled all buffers and solutions and the reaction tubes on ice.

Steps:

1. In each sample tubes, add quenching solution 20 µL.

2. In each reaction tube, add KPI buffer, the tested compound (glucuronide), and enzymes. Volumes are in the table (tubes should be placed on ice and buffers are chilled).

Final drug concentration (µM)	Enzyme working solution (1 mg/mL)	Drug stock solution (1 mM)	KPI (µL)	**Total volume (µL)**
2.5	10 µL	0.5 µL	**189.5**	**200**
5	10 µL	1 µL	**189**	**200**
10	10 µL	2 µL	**188**	**200**
20	10 µL	4 µL	**186**	**200**
40	10 µL	8 µL	**182**	**200**
80	10 µL	16 µL	**174**	**200**

3. Incubate the reaction tubes at 37 degree for 30 min.

4. Take 100 µL of samples from the reaction tube to the sample tubes accordingly.

5. Centrifuge the sample tube at 14,000 rpm for 15 min at 4 degree.

6. Inject samples in UPLC.