**Semi-continuous reaction, metabolite clean-up, and HPLC-MS analysis**

TX-TL reactions were prepared as two components (A and B) in a semi-continuous reaction as follows:

1. Component A - 100 μL of standard TX-TL reaction, in the absence of PEG, was injected into a Thermo Scientific Pierce 3.5K MWCO 96-well microdialysis device.
2. Component B - 1.5 mL SMM solution with 1 mg/mL carbenicillin was added to a 2.5 mL tube.

NOTE: Carbenicillin is supplemented to minimize microbial contamination during the reaction time course.

1. The microdialysis cassette was placed inside the 2.5 mL tube and incubated at 30 °C for 24 h with shaking (1,000 rpm).
2. Acidify samples with 1% (v/v) HCl.
3. Centrifuge at 16,000 × *g* for 30 min at room temperature.
4. Pre-equilibrate a Sep-Pak C-18 (50 mg sorbent) solid-phase extraction cartridge (Waters) with 20 mL of acetonitrile and then 20 mL of 1% (v/v) HCl.
5. Load the acidified supernatant onto the C-18 cartridge; apply pressure with a syringe or manual air pump.
6. Wash with 10 mL of 10% (v/v) ethanol.
7. Elute metabolites with 2 mL of 50% (v/v) ethanol
8. All solutions were acidified with 1% (v/v) HCl.
9. Dry samples using a vacuum centrifuge for 30 min.
10. Dissolve samples in 10 μL of MeOH and add 90 μL of 1% HCl.
11. Centrifuge at 18,000 × *g* for 30 min at room temperature.
12. Load 1 μL of supernatant onto available LC-MS for analysis. Further details of the HPLC-MS setup are provided below.

**Equipment and HPLC-MS settings used for uroporphyrin III detection**

Reaction products were analyzed with an Agilent 1290 Infinity system with an online diode array detector in combination with a Bruker 6500 quadruple time-of-flight (Q-ToF) mass spectrometer. An Agilent Extend-C18 2.1 x 50mm (1.8 μm particle size) column was used at a temperature of 40 °C with a buffer flow rate of 0.5 mL/min. LC was performed with a gradient of buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in acetonitrile). Separation was achieved using 2% buffer B for 0.6 min, followed by a linear gradient to 100% buffer B from 0.6 to 4.6 min, which was held at 100% buffer B from 4.6 to 5.6 min followed by a return to 2% buffer B from 5.6 to 6.6 min, along with 1 min post run. Spectra were recorded between a mass range of 50–1700 *m/z* at a rate of 10 spectra per second in positive polarity.