FERROCHELATASE (FECH)

Sample preparation.

Suspend ~50-µL mammalian cell pellet in 400µL TGC buffer (Tris buffered glycerol with dithiothreitol (DTT), made by dissolving 2mL or 2.52 g glycerol and 1.5mg DTT in 8.0 mL 20mM Tris pH 8.0).
Sonicate (homogenize) while in an ice bath at the lowest practicable power setting for 3 cycles x 5 sec at 50% duty (2.5 sec on, 2.5 sec off).
Determine the protein content and dilute to 1µg protein/µL with more TGD.

Ferrochelatase reaction.

Prepare three 50-µL aliquots of the cell preparation, two live and one inactivated in boiling water for 10 minutes (as control for non-enzymatic product formation).
Prepare the incubation buffer containing 160mM Tris pH 8.0, 40mM Bicine pH 8.0, 10mg/ml Tween20 and 0.38mg/mL palmitic acid),
Mix 150µL incubation buffer and 25µL zinc substrate (1mM aqueous Zn acetate) and pre-incubate for 5 minutes at 37˚C.
Mix each 50-µL aliquot of cell preparation with the 150µL incubation buffer plus zinc substrate.
Then add 25 µL of mesoporphyrin IX substrate (250µM in 160mM Tris pH 8.0, 40mM Bicine pH8.0, 2mg/ml Tween20).
Incubate the mixture for 30 min at 37˚C.
Add 750µL stop reagent (270µM ethylenediaminetetraacetic acid in a mixture containing dimethylsulfoxide-methanol, 30/70 by volume respectively).
Cool on ice for 15-20 min.
Centrifuged at 1500xg for 10 min at room temperature.

Quantitation of product Zn mesoporphyrin IX.

Inject 10µL of supernatant solution of porphyrins in 1.5M HCl into a Waters Acquity UPLC (ultra performance liquid chromatography) system, which includes a binary solvent manager, sample manager, fluorescence detector (FLR), column heater and an Acquity UPLC BEH C18, 1.7 µM, 2.1 x 100 mm column. Set the FLR for zinc mesoporphyrin IX (ZnMeso) at 406 nm excitation and 578 nm emission. Quantify the ferrochelatase product relative to a standard ZnMeso solution, also in the stop reagent. Keep the sample chamber dark and at

ambient temperature. Solvent A is 0.2% aqueous formic acid while Solvent B was 0.2% formic acid in methanol. Set the flow rate at 0.40 mL per minute at 60˚C for the total run time of 7 min. Use the following successive gradient settings for run time in minutes versus A: 0.0, 80%; 2.5, 1%; 4.5, 1%; 5.0, 80%. Keep all solvent gradients are linear.