

UROPORPHYRINOGEN DEAMINASE (UROD) ASSAY

Reaction A: Uroporphyrinogen (substrate) synthesis

Add 10 μL 0.5 $\mu\text{g}/\mu\text{L}$ rPBGD (recombinant porphobilinogen deaminase) stock to 75 μL 10mM DTT (dithiothreitol) in 0.1M Tris pH 7.65. (This is for the synthesis of uroporphyrinogen I. To make uroporphyrinogen III instead, replace 1.5 μL of the Tris/DTT with 1.5 μL of 1 $\mu\text{g}/\mu\text{L}$ recombinant uroporphyrinogen III synthase or rU3S.)

Perform subsequent steps in the dark until addition of HCl.

Start the synthesis of substrate by adding 15 μL 2.2mM PBG or porphobilinogen (0.54mg/mL) in 0.1M Tris pH7.65.

Incubate the mixture in a 37°C water bath for 35 min. (Enough rPBGD activity must have been present in the substrate synthesis step above to supply at least 30 μM uroporphyrinogen in this 200 μL activity assay.)

Neutralize the reaction mixture with 20 μL 0.15M KH_2PO_4 and cool in ice-bath for at least 2 min.

Reaction B: UROD Assay

Add 80 μL ice-cold sample to the 120 μL substrate (reaction A) solution.

Incubate the assay mixture in 37°C in water bath for 30 min.

Add the same volume (200 μL) of 3M HCl to stop the reaction.

Complete the oxidation of all porphyrinogens to porphyrins by exposing the mixture to longwave UV (320-400nm) for 30 min or under bright fluorescent light for 2h.

Centrifuge at about 16000xg in regular microfuge for 10 min.

Quantify the porphyrins by UPLC (ultra performance liquid chromatography).

For the blanks and background porphyrins, replace the substrate solution with just the Tris/DTT buffer.