**Supporting Information for**

# Direct Assay of Enzymes in Heme Biosynthesis for the Detection of Porphyrias by Tandem Mass Spectrometry. Part I. Uroporphyrinogen Decarboxylase and Coproporphyrinogen III Oxidase

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**Isolation of erythrocytes.** Human blood was drawn into a vacuum sealed tube with EDTA (BD Vacutainer, cat. no. 367899). Whole blood (3 mL) was transferred to a 15 mL plastic centrifuge tube. NaCl solution (9 mL, 0.9%, w/v) was added and the sample was centrifuged at 600 g for 10 min at room temperature. The supernatant above the erythrocyte pellet was discarded. To the pellet was added 9 mL of the above NaCl solution and the cells were re-suspended by gently inverting the tube several times followed by centrifugation. This washing step was repeated once more. To the washed erythrocyte pellet was added the NaCl solution to bring the volume to 3 mL. After re-suspending the cells, the suspension was frozen in a dry ice/acetone bath to lyse the cells. The tube was then placed in a water bath at room temperature until the solution was fully thawed. This freeze/thaw process was repeated twice. The suspension of lysed cells was centrifuged at 12,000 g for 10 min. at room temperature. The supernatant was divided into 100 μL aliquots in polypropylene microfuge tubes, which were stored at -80 °C (no loss in UROD activity was observed when frozen cell lysates were stored up to 8 months). Before use, an aliquot was thawed and diluted 10-fold with distilled water. Fifty μL of this diluted stock was used for the UROD assay following the procedure described in the main text.

**Isolation of lymphocytes.** Whole blood was collected as above and 6 mL was transferred to a plastic centrifuge tube (15 mL). The sample was centrifuged at 115 x g for 10 minutes at room temperature in a swinging bucket rotor. Much of the supernatant was removed with a plastic transfer pipet leaving about 0.5-1 ml of liquid above the top of the red cell pellet (the lymphocytes are just above the red cell layer). Using a plastic transfer pipet, the remaining liquid above the red cell layer was transferred to a 15 ml plastic centrifuge tube (up to ~1 ml of red cell layer was taken in this process). The transferred solution was diluted with PBS (GIBCO, Cat. 14190) to a final volume of 6 mL. The tube was inverted a few times to mix the solution and then the sample was gently poured above 3 mL of Ficoll Paque solution (Amersham Biosciences, cat. No. 17-1440-02) pre-loaded into a tube containing a plastic support disk (Leucosep, cat. No. 163290). The tube was centrifuged for 365 x g for 25 min at room temperature in a swinging bucket rotor. The lymphocyte layer (white band) is removed with a plastic transfer pipet and transferred to a new 15 mL plastic centrifuge tube. PBS (10 mL) is

added and the sample is placed on a vortex mixer for ~30 sec. The tube is centrifugged at 356 x g for 10 min at room temperature, and the supernatant is decanted. Cold distilled water (5 mL) is added to the lymphocyte pellet, and the sample is placed on a vortex mixer for 1 min to lyze reamaining erythrocytes. NaCl solution (5 mL, 1.8%) was added. The tube is mixed by inversion several times and centrifuged at 365 x g for 10 min at room temperature. The supernatant was decanted. The cell pellet was washed once more using 10 mL of 0.9% NaCl. After decanting the supernatant, the cell pellet was stored at -80 °C (no loss in CPO activity was observed when cells were stored frozen up to 3 months). Just prior to CPO assay, the cells (from 6 mL blood) were allowed to thaw at room temperature. NaCl (0.9 %, 0.5 mL) was added, and the suspension was sonicated at room temperature using a microtip hooked up to a Heat Systems-Ultrasonics Model W-225 sonicator (3 x ~20 sec pulses). The protein concentration in the cell lysate was measured with the Bradford dye assay (BioRad Cat. 500-0006) using bovine serum albumin as a standard. The sample was diluted to 0.4 mg/ml protein by addition of 0.9% NaCl. Fifty μL of this solution was used for the CPO assay (see main text).

**Storage of Blood and Blood Cells.** Blood was collected in the presence of various anticoagulants: 1) lithium heparin; ACD; $K_2$EDTA. The latter was found to give the highest activities of UROD and CPO after the blood was stored in anticoagulant at room temperature for 2 days. With CPO, more than 40 percent of activity was lost after two days storage at room temperature with heparin or ACD anticoagulants. EDTA could potentially allow blood analysis of samples that are shipped from other locations. When lymphocytes were stored at -80 °C, the CPO activity did not change over the period of three months. However, CPO activity was found to decrease very rapidly after sonication in an ice bath, at a rate of more than 20 percent within three to four hours. UROD is much more stable and its activity does not change on storage at room temperature for two days or at -80 °C for more than six months.

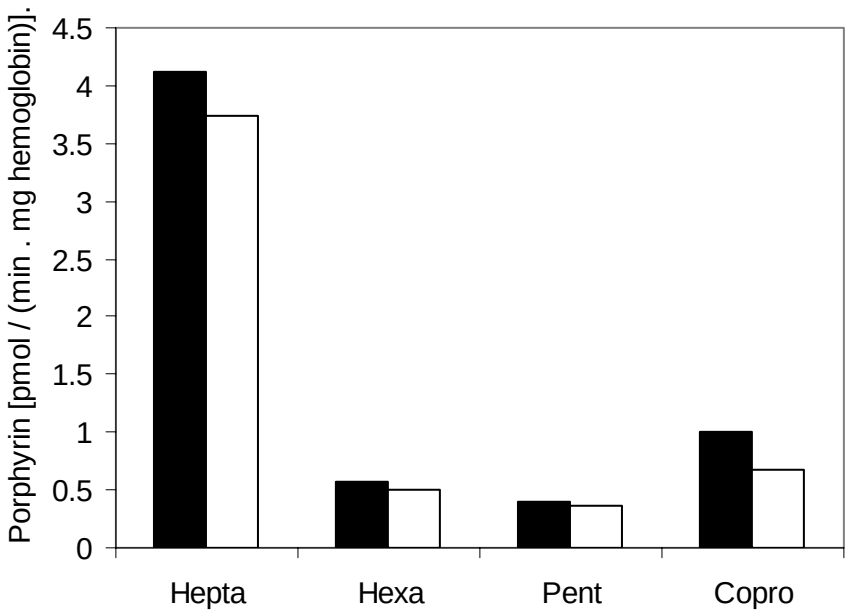


Figure S1. Activity of UROD measured in erythrocytes under nitrogen atmosphere (shaded bar) or closed cap (empty bar). Reactions were carried out at 37 °C using 8 μM uroporphyrinogen III as substrate. The assay conditions are given in the main text.

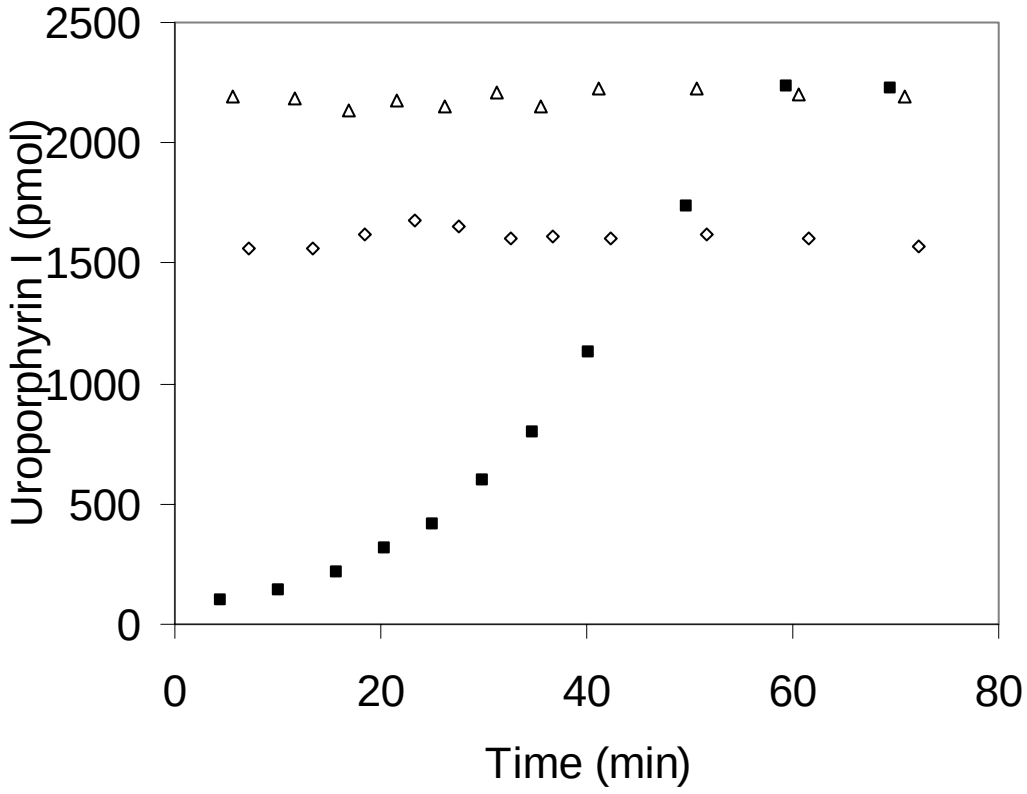


Figure S2. Time course of conversion of uroporphyrinogen III to uroporphyrin upon by spontaneous oxidation upon work up. Diamonds: Work up after incubation with red blood cell lysate and buffers; triangles: work up alone; solid squares: blank assay without blood lysate.

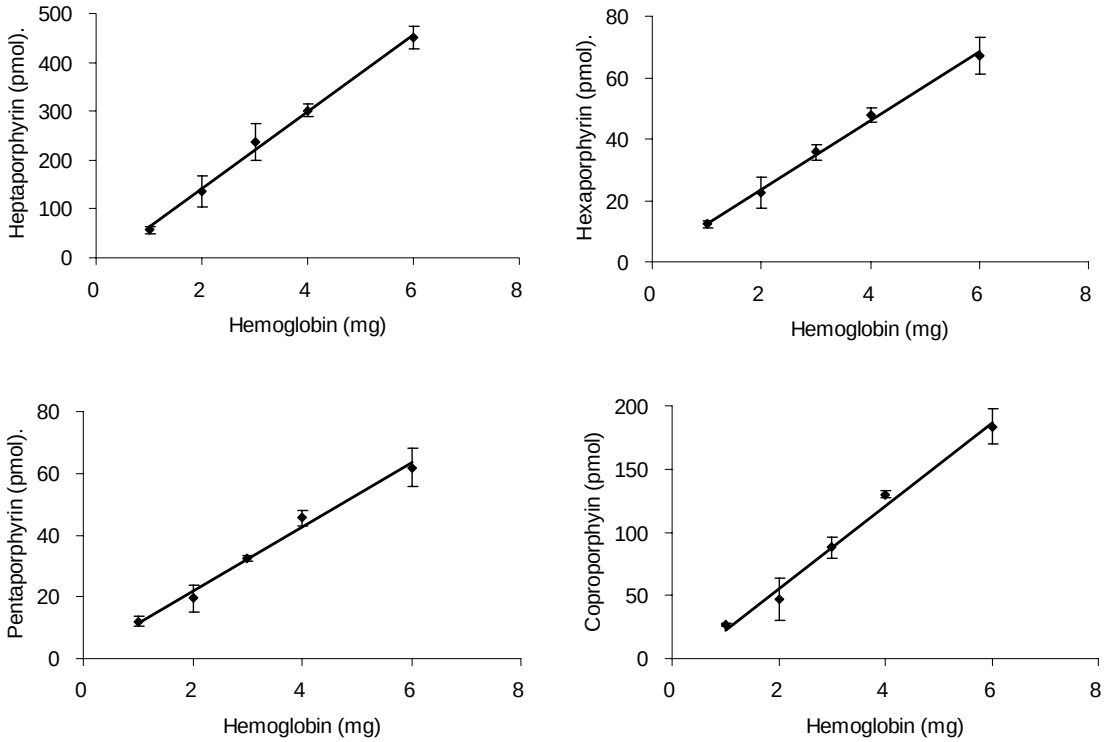


Figure S3. Activity of UROD measured in erythrocytes as a function of the amount of erythrocytes. Reactions were carried out at 37 °C using 8 μM uroporphyrinogen III as substrates and the conditions given in the main text. Error bars are shown for triplicate analyses.

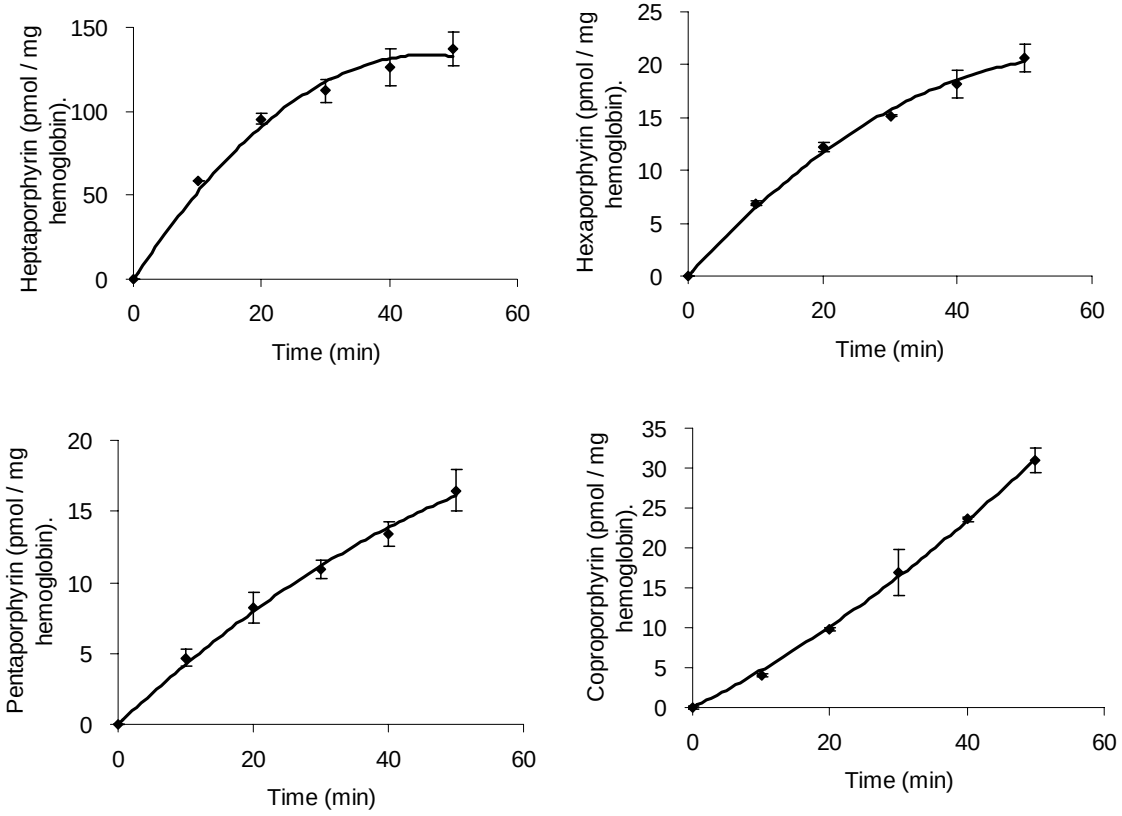


Figure S4. Activity of UROD measured in erythrocytes (3 mg hemoglobin) as a function of the incubation time. Reactions were carried out at 37 °C using 8 μM uroporphyrinogen III as substrates and the conditions given in the main text. Error bars are shown for triplicate analyses.

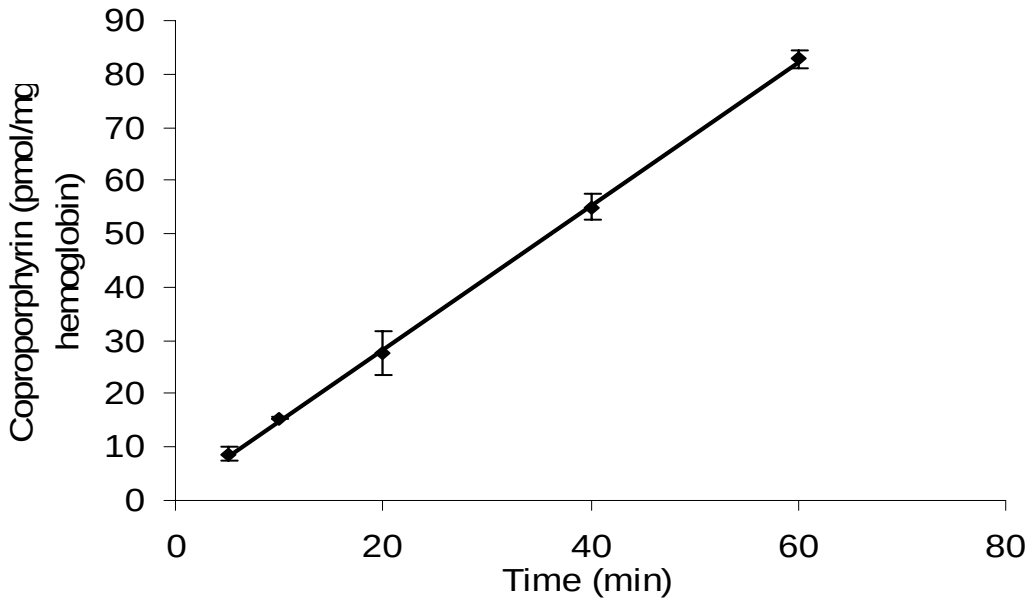


Figure S5. Activity of UROD measured in erythrocytes (1 mg hemoglobin) as a function of the incubation time. Reactions were carried out at 37 °C using 4 μM pentaporphyrinogen as substrate. The assay conditions given in the main text. Error bars are shown for triplicate analyses.

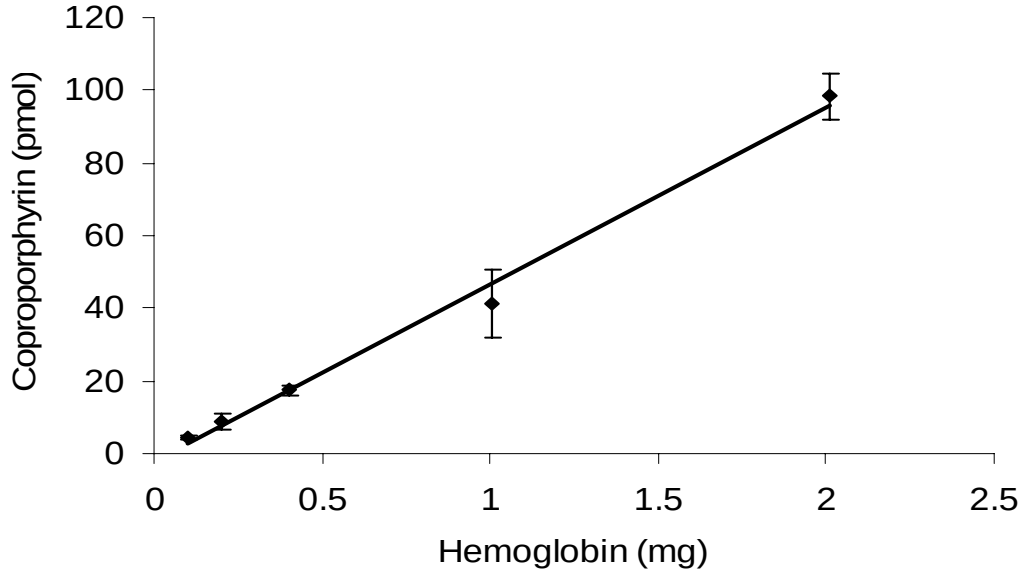


Figure S6. Activity of UROD measured in erythrocytes as a function of the amount erythrocyte hemoglobin. Reactions were carried out at 37 °C using 4 μM pentaporphyrinogen as substrate. Error bars are shown for triplicate analyses.

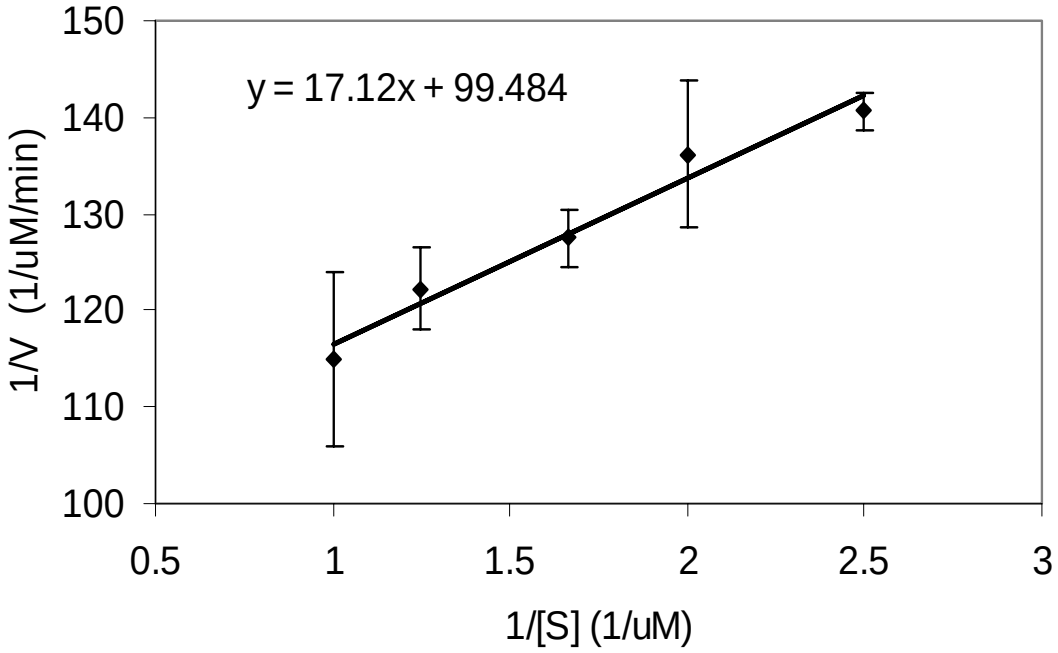


Figure S7. Activity of UROD measured in erythrocytes (1 mg hemoglobin) as a function of substrate concentration. Reactions were carried out at 37 °C using pentaporphyrinogen as substrate. Error bars are shown for triplicate analyses.

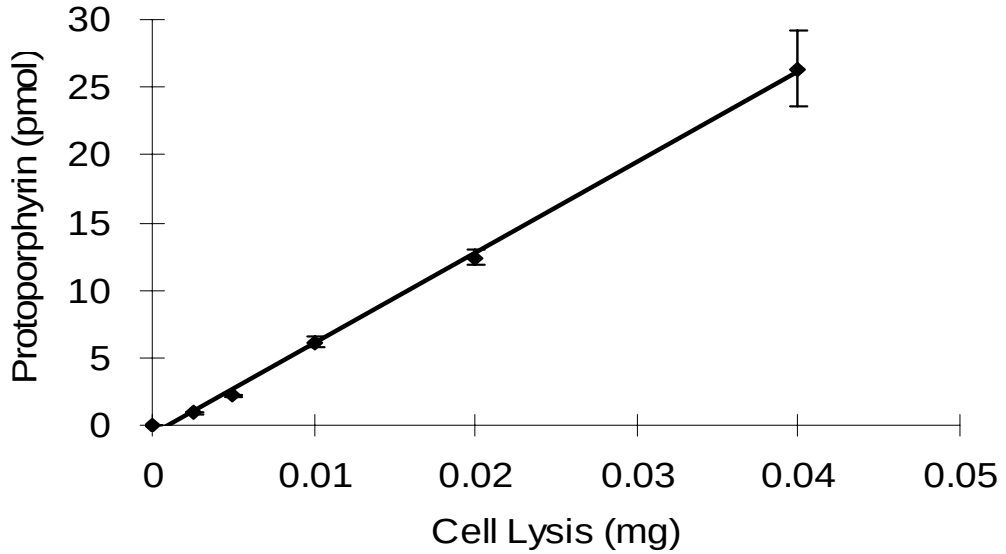


Figure S8. Activity of CPO measured in lymphocytes as a function of the amount of lymphocytes. Reactions were carried out at 37 °C using 4 μM coproporphyringoen III as substrate. Error bars are shown for triplicate analyses.

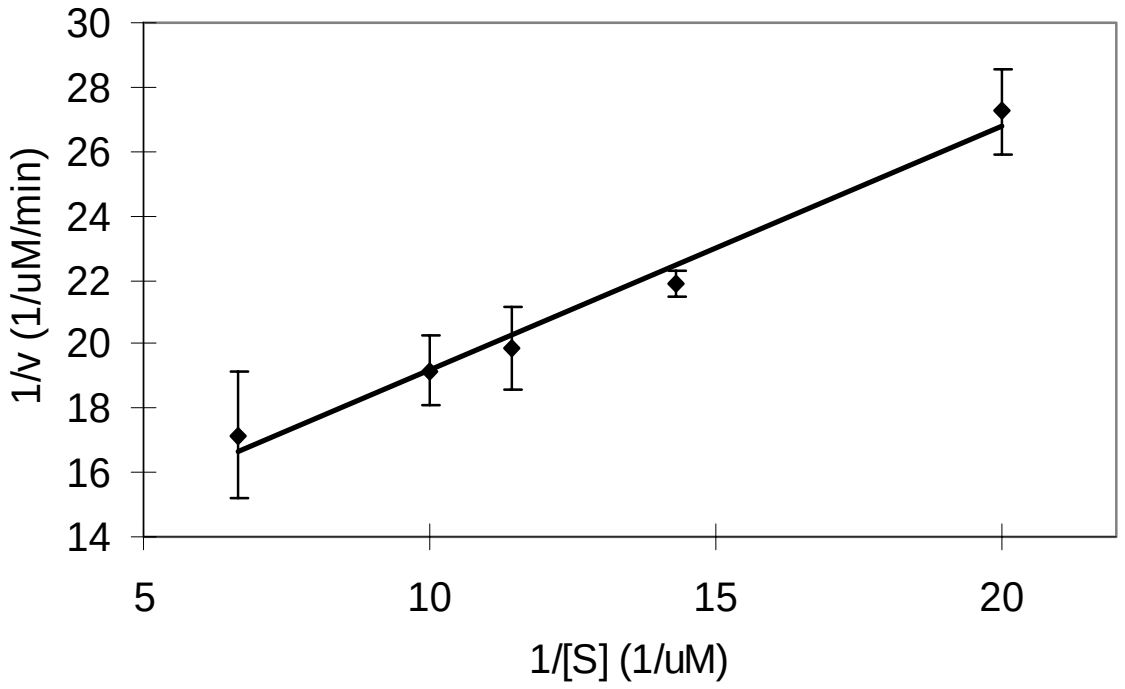


Figure S9. Activity of CPO measured in lymphocytes (0.02 mg protein) as a function of substrate concentration. Reactions were carried out at 37 °C using coproporphyringoen III as substrate. Error bars are shown for triplicate analyses.