# BLOOD UROD

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## Sample preparation

1. Draw two green-topped collection tubes of blood. These tubes have heparin as anticoagulant.
2. Spin down the RBCs (red blood cells) at 2600rpm for 5min at 4˚C.
3. Discard the plasma and buffy coat.
4. Wash RBCs twice by filling the tube with Isoton II, gently resuspending the RBC,centrifuging at 2600rpm for 5min at 4˚C and discarding the supernatant. Isoton II is a balanced electrolyte solution by Beckman- Coulter.
5. Measure the volume of packed RBCs and add 4 volumes of ice-cold H2O.
6. Stir overnight in a beaker at 4˚C with a magnetic flea, being careful not to spin fast enough to form bubbles. Keep the beaker covered with plastic wrap.
7. Spiin down the lysate at 18000rpm x 18min at 4˚C. There will be a firm pellet and a loose fluffy pellet on top of it. These are easily rendered visible by shining a red light through the centrifuge tube in in a dark room.
8. Collect the clear supernatant RBC lysate above the loose pellet.
9. Freeze the clear RBC lysate at -80˚C until ready to use.
10. Measure the hemoglobin content by running the Drabkins reaction.
11. Calculate the volume of lysate that will contain 500mg hemoglobin.

## Column preparation

1. Prepare DE52 as usual. Let stand in H2O for 1h, remove the fines. DE52 is diethylaminoethyl cellulose, pre-swollen microgranular anion exchanger by Whatman.
2. Equilibrate to pH 6.8 with 0.1M KPi (potassium phosphate) pH6.8.
3. Decrease the conductivity with 5mM KPi pH 6.8.
4. Make a slurry consisting of equal volumes of settled DE-52 resin and 5mM KPi pH6.8 buffer.
5. Pipet 6 mL (for a packed bed of about 3mL) slurry into a 12-mL syringe whose bottom is plugged with a porous polypropylene disk to retain DE-52 resin and to readily let liquids flow through.
6. Apply the RBC lysate equivalent to 500mg hemoglobin. Never let the surface of the packed bed go dry.
7. Wash with 5mM KPi pH6.8 until the eluate is colorless.

8. Allow the level of the buffer to reach the top of the column but do not allow the column to go dry. Plug the bottom of the syringe to stop the elution.
9. Add 4mL of 0.5M KCL in 5mM KPi pH6.8.  Resuspend the resin.
10. Let settle and stand for 1h in ice bath.
11. Unplug the bottom of the syringe tube and collect 4mL eluate.
12. Gently add 3mL more of 0.5M KCl in 5mM KPi pH6.8 and collect another 3mL eluate. Combine both eluates.
13. Store at -80˚C until use.

## UROD Assay

1. Prepare Reaction A for UROD assay.
2. Add 80µL of eluate to 120µL of 50mM KPi pH6.8.
3. Add 300µL Reaction A to the resulting mixture.
4. Incubate in the dark at 37˚C for 30min.
5. Add an equal volume (500µL) for 3M HCl.  Mix well.
6. 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.
7. Centrifuge at about 16000xg in s regular microfuge for 10 min.
8. Quantify the porphyrins by UPLC (ultra performance liquid chromatography).

> For the reaction blanks, replace the substrate solution with just the Tris/DTT buffer.

> For controls, assay in parallel DE-52 eluates from at least two normal and one porphyric (low UROD activity) patients.

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