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UROD Activity Assay

UROPORPHYRINOGEN DEAMINASE (UROD) ASSAY

Reaction A: Uroporphyrinogen (substrate) synthesis

1. Add 10 µL 0.5 µg/µL rPBGD (recombinant porphobilinogen deaminase) stock to 75µL 10mM DTT (dithiothreitol) in 0.1M Tris pH 7.65. (This is for the synthesis of uroporphyrinogen I. To make uroporphyrinogen III instead, replace 1.5µL of the Tris/DTT with 1.5µL of 1µg/µL recombinant uroporphyrinogen III synthase or rU3S.)
2. Perform subsequent steps in the dark until addition of HCl.
3. Start the synthesis of substrate by adding 15 µL 2.2mM PBG or porphobilinogen (0.54mg/mL) in 0.1M Tris pH7.65.
4. 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 30µM uroporphyrinogen in this 200µL activity assay.)
5. Neutralize the reaction mixture with 20µL 0.15M KH_2PO_4 and cool in ice-bath for at least 2 min.

Reaction B: UROD Assay

1. Add 80µL ice-cold sample to the 120µL substrate (reaction A) solution.
2. Incubate the assay mixture in 37˚C in water bath for 30 min.
3. Add the same volume (200µL) of 3M HCl to stop the reaction.
4. 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.
5. Centrifuge at about 16000xg in regular microfuge for 10 min.
6. 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.

UPLC of Porphyrins

ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY (UPLC) QUANTITATION OF PORPHYRINS

1. Inject 10µL of supernatant solution of porphyrins in 1.5M HCl into a Waters Acquity UPLC system which includes a binary solvent manager, sample manager, fluorescence detector, column heater and an Acquity UPLC BEH C18, 1.7 µM, 2.1 x 100 mm column.
2. Set the fluorescence detector at 404nm excitation and 618nm emission. Keep the sample chamber dark at ambient temperature. Solvent A is 0.2% aqueous formic acid while Solvent B was 0.2% formic acid in methanol.
3. Set the flow rate at 0.40 mL per minute at 60˚C for the total run time of 8 min.
4. Use the following successive gradient settings for run time in minutes versus
   - A: 0.0, 60%
   - 2.0, 20%
   - 2.5, 1%
   - 3.5, 1%
   - 3.6, 60%
5. Set the solvent composition gradient from 0.0 to 2.0 min as Waters Gradient 5 (convex with a higher slope at 0.1 min compared to that at 2.0 min).
6. Keep all other gradients are linear.

> Use solutions of known concentrations of authentic porphyrins dissolved in 1.5M HCl as standards (Porphyrin Acids, Chromatographic Marker, Product # CMK-1A, Frontier Scientific, Logan, Utah).

U3S Activity Assay

UROPORPHYRINOGEN III SYNTHASE (U3S)

Cytosolic U3S Assay

1. Mix 100 µL 20mM Tris pH 8.2, 260 µL 20mM KPi (potassium phosphate) pH 8.2 and 20 µL 0.5mg/mL rPBGD (recombinant porphobilinogen deaminase).
2. Mix well and incubate for at least 2 min at 37˚C.
3. Add 20µL 2.2mM PBG (porphobilinogen) and incubate 120±2 sec at 37˚C.
4. Add 20 µL sample previously diluted to 2 mg protein/mL with 20mM KPi pH 8.2. (Use 2mg/mL BSA or bovine serum albumin as the zero blank.)
5. Incubate for 120±2 sec at 37˚C in the dark.
6. Stop the reaction with 140 µL 6M HCL.
7. Expose the mixture to UV light for 30 min or ambient light for 2h to oxidize all porphyrinogen formed.
8. Centrifuge at 16000xg for 10 min. Collect the supernatant.
9. Quantify the porphyrins by UPLC (ultra performance liquid chromatography).

Replicates

1. Perform three reactions for each sample, a and b containing PBG, c with just the KPi buffer.
2. For each sample tube a,b,c - reagents were added at 30-sec staggered intervals, hence the 120±2 sec after the first addition of PBG.

For rhU3S protein

1. dilute U3S stock to 0.5 mg/mL with buffer consisting of 10mm Tris pH7.5, 150mM NaCl, 10% glycerol and 1mM dithiothreitol.
2. Use 2 µL of diluted stock in the assay above and 278µL KPi, not 260 µL.

Reverse FECH

- Sonicate the washed pellet of cultured bacterial cells in two volumes of 10mM KPi pH 5.5. Determine protein content and adjust to about 20mg/mL with the same buffer to prepare the homogenate. Deoxygenate all samples and reagents.
- Mix about 50µL homogenate (1.0 mg total protein) with 50 µL of assay reagent containing 100 µM hemin-imidazole, 4 mM ascorbic acid in 10 mM potassium phosphate buffer pH 5.5 under argon atmosphere.
- Cap the sample tubes securely, incubate for 1h at 45˚C in a water bath and then add 400 µL 50% v/v acetone in ethanol.
- Centrifuge the samples at 13,500 g for 10 min.
- Analyze the supernatant for PPIX fluorescence by UPLC (ultra-performance
- liquid chromatography).
- For blank controls samples of bacterial homogenates were heated for 10 min in a boiling water bath and assayed with the live samples

Pyridine hemochromogen

HEME QUANTITATIONS FROM PYRIDINE HEMOCHROMOGEN SPECTRA

Hemochromogen formation

- Prepare a pyridine reagent mix by adding 3mL 1M NaOH and 6mL pyridine (use glass pipet) to 19mL H_2O in a glass container (28mL total volume).
- Put 1000µL of the pyridine reagent in a cuvet
- Add 35µL sample containing ≥5µM hemin.
- Add 18µL aqueous 15mM $K_3Fe(CN)_6$, mix well.
- Record the oxidized spectrum at 500-650nm. Repeat until stable.
- Add 2-5mg powdered $Na_2S_2O_4$ (sodium hydrosulfite, sodium dithionite).
- Mix well under the surface of the liquid mixture (minimize mixing with air).
- Record the reduced spectrum and repeat until stable.

Calculations

- For total heme (mM) subtract the absorbance readings at 540, 556 and 575nm in the oxidized spectrum from the corresponding readings in the reduced spectrum to get ΔA540, ΔA556 and ΔA575. (For example, ΔA540 = A540 $Na_2S_2O_4$reduced - A540 $K_3Fe(CN)_6$oxidized, etc.)
- Calculate [hemin, mM] in the mixture using the extinction coefficients:
  - 20.7/mM for ΔA540 ((ΔA556-ΔA540)/20.7)
  - 32.4/mM for ΔA575 ((ΔA556-ΔA575)/32.4)
- Factor in the dilution of the sample (30x or (1000+35+18)/35) and average the two results.
- For heme a, b, or c, multiply the difference of reduced minus oxidized absorbance at the five different wavelengths below and add the resulting five multiplication products.

Reference Information

From: Berry, E.A. and Trumpower, B.L. 1987. Simultaneous determination of hemes a, b, and c from pyridine hemochrome spectra. *Analytical Biochemistry*. **161**: 1-15.

Table 4

Inverse matrix of extinction coefficients of pyridine hemochromes for calculating concentration (mM) from reduced minus oxidized absorbance at five different wavelengths.

Wavelength (nm):	540	549	558	588	620
Heme c:	-0.02778	0.04757	-0.01906	0.00084	-0.00157
Heme b:	-0.02943	-0.00088	0.04037	-0.00267	-0.00738
Heme a:	-0.02687	0.00456	0.00699	0.04353	-0.02820

Total heme may also be obtained by adding the results for hemes a, b and c for a sample. Authentic heme solutions with known concentrations may be used to check the method and also to help check that the calculations were performed correctly to obtain expected results.

Protoporphyrinogen Oxidase Activity Assay

PROTOPORPHYRINOGEN OXIDASE

Cultured cell homogenate

1. Resuspend the pellet of PBS-washed cells in about three volumes of 50mM potassium phosphate pH 7.4 (KPi).
2. 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).
3. Determine the protein concentration and adjust to 4mg/mL with more KPi.

Preparation of protoporphyrinogen

1. Prepare about 1.5mL of 1mM PPIX in an aqueous solution also containing 10mM KOH and 20% v/v ethanol in a glass test tube with a magnetic flea.
2. Put in approximately 150mg of 20% sodium in mercury amalgam (Acros Organics, Bridgewater, NJ). Cover the tube but with allowance for needle introduction and exhaust of gases.
3. Minimize exposure to light for the rest of this procedure.
4. Stir the mixture vigorously, with occasional strong shaking, for about half an hour under blowing argon gas or until all the fluorescence is gone, as monitored with minimal time exposure to a UV lamp.
5. While in an argon-filled AtmosBag (Sigma-Aldrich, St. Loius, MO), remove the solids with a syringe filter. Measure the volume of the resulting protoporphyrinogen solution.
6. Add 9 volumes of raw incubation buffer containing 120 mM Tris base, 2.5 mM EDTA free acid, 100 mM ascorbic acid and 0.12% Triton X-100.
7. Aliquot into 1.7-mL microfuge tubes and put in argon-inflated ziplock bags. Take out of the AtmosBag and store frozen.
8. Strictly follow published methods to store and then dispose of mercury.

Protoporphyrinogen oxidase reaction

1. Adjust the pH of the substrate protoporphyrinogen solution to around 8.5 with 3M HCl before use. Use pH paper indicators.
2. Add 90 µL of the pH adjusted substrate solution 10µL of sample containing 4 mg/mL protein/mL, in a 1.7mL microfuge tube. (There is no need to vortex the tubes, as this will enhance autooxidation. There is also more than enough oxygen substrate above the liquid in the tubes for the duration or the reaction.)
3. Pair each live sample with a blank, which is sample that has been heated in a boiling water bath for 10 min.
4. Incubate at 37˚C for 10 min.
5. Stop the reaction with 400µL of 30 % v/v DMSO in methanol.
6. Centrifuge at 16000xg for 10 min.
7. Immediately inject the supernatant into the UPLC, the blank and the live samples one after the other.

Quantification of product protoporphyrin IX.

1. Inject 10µL of supernatant solution of porphyrins into a Waters Acquity UPLC (ultra performance liquid chromatography) system, which includes a binary solvent manager, sample manager, photodiode array detector (PDA), fluorescence detector (FLR), column heater and an Acquity UPLC BEH C18, 1.7 µM, 2.1 x 100 mm column.
2. Set the FLR at 404 nm excitation and 630 nm emission to measure fluorescence of protoporphyrin IX (PPIX). Use the PDA to verify the absorption spectrum of PPIX peak.
3. Keep the sample chamber dark and at ambient temperature.
4. Solvent A is 0.2% aqueous formic acid while Solvent B was 0.2% formic acid in methanol. Keep the flow rate constant at 0.40 mL per minute and maintain the column at 60˚C for the total run time of 6 min. Use the following successive linear gradient settings for run time in minutes versus Solvent A: 0.0, 80%; 1.5, 1%; 3.5, 1%; 4.0, 80%.
5. For standards, use solutions of known concentrations of PPIX also dissolved in 30%DMSO in ethanol. Subtract the blank PPIX readings from every corresponding live sample.

Major reference:

Li, F., CK Lim and TJ Peters. An h.p.l.c. assay for protoporphyrinogen oxidases activity in rat liver. Biochem. J. (1987) 243, 863-866.

Porphyrinogen via Pd-Carbon

CHEMICAL REDUCTION OF PORPHYRINS USING PALLADIUM CARBON

1. Put 5.0mg Pd/C (palladium carbon) and 2.0mg porphyrin in a 13 x 100mm borosilicate test tube. Put the tube on a suitable tube rack.
2. Wet the contents with 0.2mL water to avoid potential explosion when vapor from the organic solvent comes in contact with Pd/C powder.
3. Drop a small Teflon coated magnetic flea into the tube.
4. Perform the rest of this protocol in a fume hood under minimal illumination or darkroom red light.
5. Minimize exposure to air and oxygen until actual use of the porphyrinogen.
6. Add 1.8mL methanol and place the racked mixture in a portable polyethylene glove bag (Atmosbag, Sigma-Aldrich, St. Louis, MO).
7. Replace the atmosphere inside the Atmosbag with hydrogen gas. Keep it fully inflated with hydrogen gas until after the filtration step.
8. Put the bag on top of a magnetic stirrer and keep the mixture constantly agitated until all UV fluorescence (porphyrin) has disappeared, as tested with a UV lamp. Minimize time of exposure to UV.
9. Replace the hydrogen with argon. Let the gas flow as needed to keep the bag inflated.
10. While still in the bag, remove the Pd/C powder via filtration. Transfer the mixture minus the magnet into a syringe with a glass fiber filter. Collect the filtrate into another tube. Cover the tube containing the filtrate.
11. Remove the filtrate tube from the bag, keeping it under blowing argon. Evaporate the filtrate to dryness while under blowing argon in a water bath at 60°C.
12. Return the tube containing the dry porphyrinogen into the argon-inflated gas bag and dissolve the contents in about 1mL of the appropriate deoxygenated assay buffer, but with 10mM dithiothreitol added.
13. Remove a small aliquot, put in 1.5M HCl, oxidize the porphyrinogen with UV and quantify. Adjust the rest of the deoxygenated porphyrinogen solution to the desired concentration.

1.

> Always prepare fresh on the day needed.

PBGD Activity Assay

PORPHOBILINOGEN DEAMINASE (PBGD)

PBGD Assay

1. Add 10µL homogenized sample to 75µL in 0.1M Tris pH 7.65.
2. Add 15 µL 2.2mM PBG (porphobilinogen) in 0.1M Tris pH7.65.
3. Incubate in a 37˚C water bath for 30 min.
4. Stop the reaction with 100µL 3M HCl.
5. Expose the mixture to UV light for 30 min or ambient light for 2h to oxidize all porphyrinogen formed.
6. Centrifuge at 16000xg for 10 min. Collect the supernatant.
7. Quantify the resulting porphyrins by UPLC (ultra performance liquid chromatography).

> For blanks use samples inactivated in boiling water for 10 min or live samples at 0 min incubation with PBG.

> For purified PBGD preparations, use 10µL 0.05mg/mL instead of the 10µL homogenized sample.

Iron Assay (ICP-OES)

IRON ASSAY USING ICP-OES

1. Put samples in glass vials.
2. If needed, dry under blowing argon and in 60˚C water bath.
3. Add 1mL of a mixture of 5vols of concentrated nitric acid to 2vols concentrated perchloric acid.
4. Cap loosely and put on a 100˚C heat block in a fume hood.
5. Allow to digest (wet ash) several hours to overnight.
6. Make up to 1mL with more nitric/perchloric acid. Mix well.
7. Transfer 250µL to another tube and add 1mL water for a final volume of 1.25mL.
8. Measure iron content with a Perkin Elmer 3100XT Inductively Coupled Plasma Optical Spectrometer system (ICP-OES).

> As a blank, digest 1.0mL of the nitric/perchloric acid mixture in the same batch of vials together with the samples. Subtract its raw reading from that of the samples. Any difference reading near or below 0.050ppm is not easily reproducible on this instrument.

> A standard solution of 0.500ppm of iron was used in the quantitation.

Hemin PPIX ZnPPIX Assay

HEMIN, PROTOPORPHYRIN IX AND ZINC PROTOPORPHYRIN IX

EHTYL ACETATE / ACETIC ACID EXTRACTION AND UPLC

Solvent Extraction

1. Prepare extraction solvent (EA) by mixing four volumes of ethyl acetate to one volume of glacial acetic acid.
2. Dilute sample homogenates with homogenization buffer to about 10 mg protein/mL or less.
3. Mix 50µL adjusted sample homogenate with freshly prepared 200µL EA. Vortex vigorously for one minute.
4. Centrifuge at 16000xg for 0.5 min. Collect the resulting supernatant, which is around 90% of the total volume.
5. Quantify by UPLC (ultra performance liquid chromatography).

UPLC Quantitation

1. Inject 10µL of the supernatant solution above into a Waters Acquity UPLC system which includes a binary solvent manager, sample manager, photodiode array detector (PDA), fluorescence detector (FLR), column heater and an Acquity UPLC BEH C18, 1.7 µM, 2.1 x 100 mm column.
2. Set the PDA to measure hemin absorbance at 398nm and the FLR to measure fluorescence of protoporphyrin IX (PPIX) at 404 nm excitation and 630 nm emission and of Zn protoporphyrin IX (ZnPPIX) at 406 nm excitation and 586 nm emission.
3. Keep the sample chamber dark and at ambient temperature.
4. Solvent A is 0.2% aqueous formic acid while Solvent B was 0.2% formic acid in methanol.

5. Set the flow rate at 0.40 mL per minute at 60˚C for the total run time of 12 min.
6. Use the following successive gradient settings for run time in minutes versus A:
   - 0.0, 80%
   - 7.5, 1%
   - 9.5, 1%
   - 10.0, 80%.
7. Set the solvent composition gradient from 0.0 to 7.5 min as Waters Gradient 5 (convex with a higher slope at 0.0 min compared to that at 2.0 min).
8. Keep all other gradients are linear.

> For standards, extract solutions of known concentrations of authentic hemin, PPIX and ZnPPIX dissolved in 1% aqueous trimethylamine or 0.1M NH_4OH.

> For greater sensitivity set FLR to single detection only (hemin or ZnPPIX, not both).

FECH Activity Assay

FERROCHELATASE (FECH)

Sample preparation

1. Suspend ~50-µL mammalian cell pellet in 400µL TGD 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)
2. 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)
3. Determine the protein content and dilute to 1µg protein/µL with more TGD

Ferrochelatase reaction

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

Quantitation of product Zn mesoporphyrin IX

1. Inject 10µL of supernatant solution of porphyrins 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.
2. Set the FLR for zinc mesoporphyrin IX (ZnMeso) at 406 nm excitation and 578 nm emission.
3. Quantify the ferrochelatase product relative to a standard ZnMeso solution, also in the stop reagent.

4. Keep the sample chamber dark and at ambient temperature.
5. Solvent A is 0.2% aqueous formic acid while Solvent B was 0.2% formic acid in methanol.
6. Set the flow rate at 0.40 mL per minute at 60˚C for the total run time of 7 min.
7. 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%
8. Keep all solvent gradients are linear.

COPOX Activity Assay

COPROPORPHYRINOGEN III OXIDASE (COPOX) ASSAY

Reaction A: Uroporphyrinogen III synthesis

1. Add 8 µL 0.5 µg/µL rPBGD (recombinant porphobilinogen deaminase) stock and 2µL 1µg/µL rU3S (recombinant uroporphyrinogen III synthase) to 70µL 10mM dithiothreitol in 0.1M Tris pH 7.65.
2. Perform subsequent steps in the dark until the addition of HCl.
3. Start the synthesis of substrate by adding 15 µL 2.2mM PBG (prophobilinogen) in 0.1M Tris pH7.65.
4. Incubate the mixture in a 37˚C water bath for 35 min.
5. Neutralize the reaction mixture with 20µL 0.15M KH_2PO_4 and cool in an ice bath for at least 2 min.

Reaction B: Coproporphyrinogen III synthesis

1. Add 75µL of a mixture containing 10µg rhUroD (recombinant human uroporphyrinogen deaminase) and 8µg bovine serum albumin in 50mM KPi (potassium phosphate) pH6.8 to Reaction A.
2. Incubate at 37˚C for 1h in the dark.
3. Adjust the pH to 7.5-8.0 with about 0.3µL 4M KOH.

Reaction C: COPOx assay

1. Add 50µL of 0.2µg protein/µL sample in homogenization buffer to Reaction B.
2. Incubate at 37˚C water bath for 30 min.
3. Add 80µL 6M HCl to stop the reaction.
4. 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.
5. Centrifuge at about 16000xg in regular microfuge for 10 min.
6. Quantify the porphyrins by UPLC (ultra performance liquid chromatography).

> Use solutions of known concentrations of authentic porphyrins dissolved in 1.5M HCl as standards (Porphyrin Acids, Chromatographic Marker, Product # CMK-1A, Frontier Scientific, Logan, Utah). Make similar solutions of protoporphyrin IX for standard quantitation.

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Coproporphyringen III substrate may also be chemically synthesized from coproporphyrin III in the presence of palladium carbon and hydrogen gas.

Colorimetric PBG Assay (Ehrlich)

COLORIMETRIC PORPHOBILINOGEN ASSAY (EHRLICH)

Modified Ehrlich Reagent

1. Dissolve 1.0 gm p-dimethyl aminobenzaldehyde in 30mL glacial acetic acid.
2. Add 8.0 mL 70% perchloric acid.
3. Bring to 50 mL with glacial acetic acid.
4. Store in the dark at 4˚C for about 2 weeks.

Porphobilinogen (PBG) Assay

1. Mix 2.0mL sample with 2.0mL fresh modified Ehrlich reagent.
2. Measure absorbance at 555nm.

Standard PBG

1. Prepare 0.54mg/mL (2.2mM) PBG stock in 0.1M Tris pH 7.65.
2. (Store at -20˚C until needed.)
3. Dilute 1:1000 in H_2O.
4. Mix 2mL with 2mL fresh modified Erlich reagent.
5. Measure absorbance at 555nm.
    - 1mM PBG = 61 abs units at A555.

BLOOD UROD

BLOOD UROD

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.

Blood Hemoglobin Assay (Drabkins)

BLOOD HEMOGLOBIN ASSAY (DRABKINS)

Drabkins Reagent

1. Dissolve 1.0g $NaHCO_3$, 0.05g KCN and 0.20g $K_3Fe(CN)_6$ in some water.
2. Bring to 1.0L final volume.
3. Store refrigerated.

Hemoglobin Assay (formation of cyanomethemoglobin)

1. Pipet 5.0mL Drabkins reagent into a test tube.
2. Add 20µL of red blood cell (RBC) lysate.
3. Mix well and let stand 10min to 1h.
4. Measure absorbance at 540nm.
5. Duplicate samples should be within 0.01 AU of each other, otherwise, repeat.
6. Use 20µL water instead of RBC lysate as zero blank.

> E = 0.683 mL/mg
> Concentration = (A540 / E)*dilution = mg/mL
> Gm/100mL = gm%

> The 20µL added to 5 mL makes a 251 dilution, appropriate for whole blood or lysates diluted up to 1/5; dilute further in case needed.

ALAS Activity Assay

d-AMINOLEVULINIC ACID SYNTHASE (ALAS)

Mouse liver homogenate

1. Weigh out ~100mg liver.
2. Add four volumes (400µL) of ice-cold 50mM potassium phosphate (KPi) pH7.4.
3. Homogenize with ten up-and-down strokes in a 2-mL glass-Teflon (Potter- Elvehjem) tissue homogenizer in an ice bath.
4. Store the resulting 20%w/v homogenate at −80 °C until needed.

Cultured cell homogenate

1. Wash the cells with phosphate buffered saline (PBS) pH 7.4.
2. Resuspend in about three pellet volumes of 50mM potassium phosphate (KPi) pH7.4.
3. 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).

Preparation of succinyl coenzyme A

1. Prepare these three aqueous solutions and keep on ice:
   - 23.85 mg/mL coenzyme A
   - 10.5 mg/mL succinic anhydride, ground to fine powder
   - 25 mg/mL $NaHCO_3$.
2. Mix equal volumes of these solutions and incubate on ice for 30 min.
3. Use in the ALAS assay.
4. The resulting ~10mM succinyl CoA solution could be also be aliquoted, stored for more than three months at −80 °C and thawed once, only when needed.
5. Succinic anhydride must be dissolved in ice-cold water to minimize conversion to succinic acid before reacting with coenzyme A. Check that the final mixture reaction is maintained at pH 7–7.5 by the $NaHCO_3$ solution.

ALAS assay

1. Adjust enough of each sample to 5-10 mg protein per mL with 50mm potassium phosphate (KPi) pH7.4 to make 6 x 25-µL aliquots for three pairs of live and blank replicates. Blanks are heat inactivated for 10 min in a boiling water bath before the

addition of the ALAS assay buffer, or incubated at 0 min at 37˚C after the ALAS assay buffer has been added.
2. For each 25-µL of sample make 25µL of ALAS assay buffer by mixing 15.5µL 50mM KPi pH 7.4, 2.5µL 1M aqueous glycine pH~7, 2.5µL 10mM succinyl CoA, 0.5µL aqueous succinylacetone and 4.0µL 1mM aqueous pyridoxal 5'-phosphate.
3. Add 25 µL ALAS assay buffer to each 25-µL aliquot of the protein adjusted sample.
4. Incubate the resulting mixture at 37 °C for 30min.
5. Add 450 µL of ice-cold water.
6. Keep the diluted ALAS sample on ice or store frozen until the ALA can be derivatized.

Derivatization of d-aminolevulinic acid (ALA)

1. Prepare derivatizing agent (DA) each sample by mixing water, 37% formaldehyde, ethanol and acetylacetone in a ratio of 107:5:15:23 by volume, respectively. Stir/vortex vigorously for 3 min or more until a clear colorless solution is obtained.
2. Carry out all subsequent steps under minimal lighting.
3. Mix 50-µL aliquot of the diluted ALAS assay sample (above) with 150 µL DA.
4. Incubate in a covered heat block at about 100–103 °C for 5.0min.
5. Cool immediately in an ice bath for 1 to 6h.
6. Centrifuged for 10 min at 14,000 rpm in a microfuge at 4 °C.
7. Collect the supernatant.
8. Keep at 4°C until injection into the UPLC.

Ultra performance liquid chromatography (UPLC) quantitation

1. Inject 10µL of supernatant containing the derivatized ALA into a Waters Acquity UPLC system which includes a binary solvent manager, sample manager, fluorescence detector, column heater and an Acquity UPLC BEH C18, 1.7 µM, 2.1 Å~ 100mm column.
2. Set the fluorescence detector at 370nm excitation and 460nm emission.
3. Keep the sample chamber dark and at 5 °C. Solvent A is 0.2% aqueous formic acid while Solvent B is 100% methanol.
4. Set the flow rate at a constant 0.3 mL/min and the column at 50°C for the total run time of 12min.
5. Use the following gradient schedule with the percent Solvent A at each step as follows:
    - 0 min, 80%
    - 6 min, 60%
    - 7 min, 1%
    - 9 min, 1%
    - 9.5 min, 80%
6. Set the gradient for solvent composition from 0 to 6 min as Waters Gradient 5 (convex with a higher slope at 0 min compared to that at 6 min), and that from 6 to 7 min at Waters Gradient 7, concave.
7. Keep all other gradients in the method linear.

Standard curves

1. Prepare pairs of sample aliquots at the same protein concentration as the unknowns to contain authentic ALA at seven different concentrations in a range similar to those as the sample unknowns, including 0.0µM.
2. Inactivate one of each pair.
3. Put these spiked samples through the ALAS activity assay protocol.
4. Subtract the background UPLC peak readings at 0.0 µM was from those of the spiked samples and use these results to construct a standard curve that to determine the [ALA] in the 25-µL sample aliquots.

ALAD Activity Assay

AMINOLEVULINIC ACID DEHYRATASE (ALAD)

Mouse liver homogenate

1. Weigh out ~100mg liver.
2. Add 400 µL 1mM DTT (dithiothreitol) in 50mM KPi (potassium phosphate) pH 6.8.
3. Homogenize with ten up-and-down strokes in a 2-mL glass-Teflon (Potter- Elvehjem) tissue homogenizer in an ice bath.
4. Store the resulting 20%w/v homogenate at −80 °C until needed.

Cultured cell homogenate

1. Wash the cells with phosphate buffered saline (PBS) pH 7.4.
2. Resuspend in about three pellet volumes of 50mM KPi pH6.3 for mammalian cells, or 200mM glycine/NaOH pH 9.2 for yeast.
3. For mammalian cells, 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).
4. Sonicate yeast cells at the highest power setting that does not cause significant aerosolization of the mixture for 12 5-sec cycles.
5. Store the resulting homogenate at −80 °C until needed.

ALAD Assay

1. Prepare two replicate pairs for each sample, two live and two heat-deactivated for 10 min in boiling water (for use as blank).
2. Prepare similar solutions of PBG (porphobilinogen, ALAD product) for use as standard curve.
3. Add 175µL solution containing 1mM DTT and 1.714mM d-aminolevulinic acid or ALA (for a final 1.5mM in 200µL) to 25µL (500µg protein) sample or standard PBG.
4. Incubate at 37˚C for 30 min.
5. Add 400µL of 100mM Tris pH 7.6 containing 150µM succinylacetone (SA) and 1 mg/mL recombinant porphobilinogen deaminase (PBGD). (The Tris adjusted the system to near pH 7.6 for optimal PBGD activity, while the SA inhibited further ALAD activity.)
6. Incubate at 37˚C for 45 min.
7. Add 200µL of 6 M HCl to stop the reaction.

8. Expose the mixture to UV light for 30 min or ambient light for 2h to oxidize all porphyrinogen formed.
9. Centrifuge at 16000xg for 10 min. Collect the supernatant.
10. Quantify the porphyrins by UPLC (ultra performance liquid chromatography).