Laboratory Procedure Manual

Analyte: **Erythrocyte Protoporphyrin**

Matrix: **Whole Blood**

Method: **Modification of the method of Sassa et al.**

Method No.: 0164A

Revised:

as performed by: *State of New York Department of Health*
Wadsworth Center
Trace Metals Laboratory

Contact: *Patrick Parsons, Ph.D.*

Important Information for Users

New York Department of Health periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for NHANES 2003–2004 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
l39_c	LBDEPPSI	Protoporphyrin (µmol/L RBC)
	LBXEPP	Protoporphyrin (µg/dL RBC)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Porphyrins and heme components are extracted from whole blood into a 4:1 mixture of ethyl acetate-acetic acid. Porphyrins are then separated from heme by back-extraction into a 1.5 M hydrochloric acid solution, and quantitatively determined by molecular fluorometry using a spectrofluorometer calibrated with protoporphyrin IX (PPIX) standard solutions; however, the exact concentration of the standards must first be established using molecular absorbance, Beer's Law, and the millimolar absorptivity of PPIX.

The analytical method for erythrocyte protoporphyrin (EP) routinely employed by the EP Lab is based largely on those originally described by Sassa et al. (1973) and Chisolm and Brown (1975). New York State's extraction method owes much to contributions from other public health labs, including the CDC, and closely follows the key elements of the consensus method for EP as published by the National Committee for Clinical Laboratory Standards (NCCLS C42-A*, 2001). At the invitation of Dr. Sassa, the EP Laboratory's routine method for EP was published as Unit 8.8 in Current Protocols in Toxicology, 1999 by J. Wiley & Sons, Inc. Elements of this protocol are reproduced below, but a reprint of the original publication is available from the EP lab director.

2. SAFETY PRECAUTIONS

Wear gloves, lab coat, and safety glasses when handling human blood specimens. Place all plastic tips, sample cups, and gloves that contact blood in a biohazard waste container.

> Dispose of all biological samples and diluted specimens in a biohazard waste container at the end of the analysis.

3. COMPUTERIZATION, DATA SYSTEM MANAGEMENT

Clinical specimens received into the EP lab for analysis are pre-accessioned for analysis by assigning each one a unique code number based on the calendar year (four digits) + the Julian Day (three digits) + a four digit number for the day's sample, for a total of 11 digits. Bar-coded specimen labels are generated and affixed to each sample. Each specimen is tracked electronically via its unique accession number in the EP lab Laboratory Information Management System (LIMS) software, a customized database for tracking specimens, ordering tests, reviewing, and releasing results. The LIMS software is installed on a Novell Netware Server. The LIMS records the batch identification number (ID) that the specimen is allocated to, individual replicate results, final reported result, the specimen receipt date, analysis date and date final results are reported. The system is backed up daily both to a digital audio tape (DAT) and by mirroring the hard drives to another server located at a remote site for catastrophic backup protection. Within the EP lab, each analytical instrument, including the Perkin-Elmer LS50B spectrofluorometer, is interfaced to the LIMS via ethernet. At the conclusion of the analysis, the tech reviews all quality control (QC) data and, if in control; analytical results are uploaded to the LIMS electronically. This minimizes transcription errors on the part of the analyst. The LIMS software automatically orders repeat tests if the QC rules are satisfied. Results must be reviewed and released by the technician and then reviewed and released by the supervisor before a report can be generated. For the NHANES project, the analytical data would be transferred to a spreadsheet pending file transfer to the appropriate contact. For the purposes of this proposal, QC data for each batch of results generated for NHANES would also be reviewed by the EP Lab Director.

Since the EP lab local access network (LAN) is directly connected to several databases within Wadsworth, a direct ethernet connection to the Internet is not permitted. Access to the Internet is limited to a dial up connection into the Wadsworth.org server because of confidentiality requirements placed on us by the Department of Health (DOH). However, for blood EP results, the LIMS system produces results as an ASCII file of standardized format which is uploaded to the DOH via a secure web site within the DOH using the dial up connection.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

Either venous or capillary whole blood is suitable for this analysis. Blood must be preserved in either EDTA (preferred) or heparin. The latter forms fibrin clots more easily; therefore, EDTA is preferred for long-term storage.

For duplicate analyses a minimum of 100 µL is required (50 µL for each replicate analysis). If further testing is required to resolve poor agreement between replicates or to repeat the analysis for elevated results, an additional 100 µL is required. To account for losses during the pipetting stage, the minimum amount of specimen required is 150 µL. The optimal volume required to meet current QC guidelines and to ensure that several representative samples can be removed and analyzed would be 1 mL. Analyses were conducted using the extraction method. However, blood specimens for EP testing must be protected from exposure to light by wrapping the tube in aluminum foil. Specimens can be transported by USPO, FedEx, or UPS at ambient temperature without a problem.

Tests requiring whole blood with a preservative cannot be performed on a serum or plasma specimen.

Whenever it is determined that a specimen volume is less than the minimum volume required to perform the indicated test, it should be reported as “QNS” (quantity not sufficient) in the accessioning book and in the result codes field of the LIMS system.

If the sample is clotted, report as “CLOT” in the accessioning book and in the result codes field of the LIMS system.

Whole-blood specimens may be analyzed for EP up to 8 weeks after collection, even when stored at 22°C, according to the published literature (Wang and Peter, 1985). In-house stability studies have confirmed this and have shown specimen stability up to 10 weeks for red blood cell (RBC) protoporphyrin when stored at 4°C. However, for practical public health purposes, it is desirable for venous blood specimens to be analyzed within 2 weeks of collection.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

A. Solutions and Reagents

5-µg protoporphyrin IX tube standards (Porphyrin Products, Logan UT).

Protosolv (Porphyrin Products, Logan UT).

Ethyl acetate (GC spectrophotometric grade, eg., Omnisolv or equivalent).

Glacial acetic acid (Trace metal grade, eg.,Baker InstraAnalyzed grade or equivalent).

1.5 M hydrochloric acid (Trace metal grade, e.g., Baker InstraAnalyzed grade or equivalent).

B. Special Equipment

(1) Perkin-Elmer LS 50B Spectrofluorometer equipped with a red-sensitive photomultiplier tube (Hamamatsu R 928) and a cell holder modified to accommodate standard 10-mm × 75-mm glass culture tubes.

(2) Beckman Model DU70 ultra-violet-visible absorption spectrophotometer equipped with standard 1-cm^2 quartz cells.

10-mm × 75-mm borosilicate glass culture tubes (Kimble Corning or Scientific Products).

One 1-L and 250-mL graduated cylinders.

10-mL glass volumetric class A pipette.

Adjustable micropipette in the range 400–1600 µL.

Three fixed-volume micropipets: 100-µL, 400-µL, and 500-µL.

Two 1-L actinic glass bottle-style pipettes, e.g., Repipet, Barnstead/Thermolyne, or equivalent with 1-mL glass dispensers. (Note: all plastic materials, i.e., uptake tubes and/or dispensing tips, must be replaced with glass devices to ensure the integrity of the EtOAc-HAc reagent. This may require the services of a glassblower to modify commercial available devices)

One 1-mL and five 2-mL glass volumetric class A flasks wrapped in foil.

Laboratory vortex mixer.

Small bench-top laboratory centrifuge suitable for accommodating 10-mm × 75-mm glass culture tubes, e.g., Sero-Fuge, Clay Adams, or equivalent.

10-mL evacuated specimen collection tubes with EDTA anticoagulant (e.g., purple cap Vacutainers Becton Dickinson or equivalent).

Microplate with 0.5-mL wells

Light-tight cabinet or box suitable for storing a rack(s) of culture tubes containing extracted standards and samples, protected from prolonged exposure to bright light.

Photographic dark room fluorescent tube filters, e.g., Macolite Toob Gard or equivalent.

C. Prepare PPIX calibration standard stock solution.

(1) Reconstitute 5-µg of protoporphyrin IX by adding 100 µL of Protosolv to the vial. Mix the contents gently without inverting the tube. Place the tube in a darkened cabinet for 10 minutes to allow complete dissolution of the material. The vial should be capped to reduce losses of volatile components due to evaporation.

(2) Add 10 mL 1.5 M HCl to the vial using a glass pipette and mix the contents by inverting the tube. The concentration of protoporphyrin IX is approximately 50 µg/100 mL.

NOTE: Several alternatives, protoporphyrin IX dimethyl ester or the protoporphyrin IX disodium salt, may be used in place of the 5 µg PPIX standard. They are available from Porphyrin Products, Logan, UT, or from Sigma Chemical Company, St. Louis MO. Their exact concentration must be standardized using molecular absorbance too as described below. Protoporphyrin IX dimethyl ester is hydrolyzed before use (Gunter et al., 1989).

NOTE: Some laboratories use coproporphyrin I or coproporphyrin III (Porphyrin Products, Logan, UT) as secondary standards because of their greater stability compared to PPIX. Equivalent "protoporphyrin IX" values are assigned to these solutions, which are used to calibrate the spectrofluorometer on a daily basis. However, a correction factor for each new batch of coproporphyrin materials must be determined using PPIX.

D. Measure the molecular absorbance of PPIX in the stock solution.

(1) Fill a 1-cm^2 quartz spectrophotometer sample cell with an aliquot of the ~50 µg/100-mL PPIX stock solution. Fill a 1-cm^2 spectrophotometer reference cell with an aliquot of 1.5 M HCl.

(2) Place the sample and reference cells in an absorbance spectrophotometer and scan the spectrum between 395–420 nm. Record the maximum absorbance (λ_{max}), A, of the band relative to HCl.

Beckman DU70

(3) Turn on both UV and VIS lamps. Press SCAN to initiate program (420–395 nm). Press START. Insert clean quartz cuvette containing 1.5 M HCl (Reagent #2). Remember, the light beam passes from left/right, so the frosted sides of the cuvette need to face front/back. Press RUN to zero instrument.

(4) Insert cuvette containing PPIX Stock Standard. Press RUN to read sample. Move X-hair to peak top (highest absorbance) and press COPY to print peak and maximum absorbance reading. On the printed copy of the scan, note that the highest wavelength/absorbance value is printed on the LOWER RIGHT side of the copy under the word TRACE.

NOTE: The exact mass of PPIX in the tube is not known, and is only nominally estimated at 5 µg. Therefore, the molecular absorbance of PPIX in solution is required to calculate the true concentration of the stock solution from Beer's Law.

Calculate the exact stock concentration of the PPIX using molecular absorbance:

Beer's Law: $A = m\varepsilon cl$

Where :
- A = absorbance (measured)
- $m\varepsilon$ = millimolar absorptivity (241 L $mmol^{-1}$ cm^{-1})
- c = PPIX concentration in mmol/L (unknown)
- l = cell path length in cm (1 cm)

PPIX relative molecular mass = 562.3 g/mol.

By rearrangement:

$$c\ (\text{mmol / L})\ \frac{A}{m\varepsilon\ l} = \frac{A}{241}$$

$$c\ (\mu\text{g / mL}) = A\ \frac{562.3}{241} = A \text{ x } 2.33$$

$$c\ (\mu\text{g / 100 mL}) = A \text{ x } 233$$

SPECIAL NOTE: The mε value was adopted in the early days of EP testing and was based upon the best available data at the time. Although the correct mε value is now known to be 297 L $mmol^{-1}$ cm^{-1} (Gunter et al., 1989), the 241 value is still widely used. See the commentary below for a more detailed discussion of this issue. Unfortunately, it has not been possible to change the long-standing practice of routine clinical laboratories and proficiency testing programs in the US that continue to use the 241 value to standardize their EP results. Because the NHANES database has been standardized using 297, all EP results reported as part of this proposal will have to be corrected to 297 to ensure comparability with the previous NHANES data. To avoid confusion at the bench level, all analyses for EP will be generated using 241. Conversion from 241 to 297 requires only a simple multiplication, EP(241) × 0.811 = EP(297), which can be applied in the final report.

E. Prepare working standards by serial dilution of the stock solution.

Wrap five 2-mL volumetric flasks in aluminum foil to protect the solutions from light. Using an adjustable micropipette, transfer 1600-µL, 1200-µL, 800-µL and 400-µL aliquots of the stock solution into four of the 2-mL flasks labelled 40 µg/100 mL, 30 µg/100 mL, 20 µg/100 mL, and 10 µg/100 mL respectively; the fifth flask serves as the BLANK or zero standard.

Dilute each 2-mL flask to volume using 1.5 M HCl. This yields standards S_t, S_3, S_2, S_1, and S_b respectively, where S_t = top standard and S_b = blank.

Transfer 50-µL of each PPIX standard into duplicate 10 × 75 mm glass culture tubes. Prepare a third culture tube containing the top standard, S_t, for adjusting and optimizing the spectrofluorometer.

F. Calculate the exact concentration of each PPIX working standard:

STD	Stock Concentration, µg/100 mL			Dilution Factor		Final Working Concentration, µg/100 mL
S_t	233 x A	x	0.8	(1600 µL / 2 mL)	=	~40
S_3	233 x A	x	0.6	(1200 µL / 2mL)	=	~30
S_2	233 x A	x	0.4	(800 µL / 2 mL)	=	~20
S_1	233 x A	x	0.2	(400 µL / 2 mL)	=	~10
S_b	233 x A	x	0.0	(0 / 2 mL)	=	0

Where A is the maximum absorbance value, λ_{max}, obtained from step E above.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Ensure that the excitation lamp is properly aligned, and set the slit width to 2.5 nm and adjust the gain accordingly.

NOTE: slit widths wider than 5 nm may give falsely elevated EP results. This has been observed in our laboratory by measuring the fluorescence intensity of blood extracts and PPIX solutions at 2.5, 5, and 10-nm spectral slit widths. In one case, a 38% increase in fluorescence in blood relative to standards was recorded as the slit width was increased to 10 nm. Some blood samples have shown only a minor increase in FI, while others show no effect.

Set the instrument's excitation wavelength to 408 nm, and use the extra top standard tube, St, to scan the emission wavelength from 645–680 nm to locate the precise wavelength for maximum intensity.

NOTE: a scan of the PPIX fluorescence spectrum shows two bands: one at 662 nm and another at 607 nm. The 607-nm band is more intense than the one at 662 nm, which is broader and, while a little less-sensitive, is much easier to peak. One consequence of the improved sensitivity of the 607-nm band is worse precision and, since sensitivity is not a critical issue in this analysis, the improvement in precision at the 662-nm band is preferred.

Discard the standard tube used to set up the instrument. Use one of the blank tubes, S_b, to 'blank adjust' the instrument to zero. Check to see if the zero standard has no fluorescence (relative to deionized water). If it does, the reagents may contain interfering fluorescent materials. Analyze standards in duplicate and in the sequence S_b, S_1, S_2, S_3, S_t. Run any secondary standards or quality control materials following the primary standards. Ensure that the results for any QC materials are within acceptable ranges before continuing with blood samples for clinical testing.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Prepare blood specimens and quality control samples

(1) Bring all blood specimens and QC samples to room temperature and mix by inversion or by placing them on a laboratory rotator prior to dilution.

(2) Transfer 200 µL of deionized water into a diluting well. Using a micropipette, withdraw a 50-µL aliquot of well-mixed whole blood. Remove any excess blood adhering to the pipet tip by carefully wiping it with a laboratory tissue. Be careful not to touch the open end of the tip. Deposit the 50-µL blood sample into the dilution well containing 200 µL of water and mix thoroughly by withdrawing and dispensing, i.e., pumping, the blood-water sample several times until the solution in the tip appears clear.

(3) Using a clean tip, transfer 50 µL of the diluted blood from the well into a 10-mm × 75-mm glass culture tube. Prepare all blood specimens and QC samples in duplicate.

B. Extract calibration standards, blood specimens and QC samples.

(1) Using a glass bottle-style pipette, add 1 mL of EtOAc-HAc reagent to the glass culture tubes containing standards, QC materials and blood samples. Thoroughly mix for 10 sec. using a vortex mixer. To ensure complete mixing, hold the glass tube at the top during vortexing.

(2) Centrifuge the glass culture tubes for 3 min. at 1000 × *g* (or ~3000 rpm for a rotor radius of 10 cm).

(3) Decant the supernatant into a clean glass culture tube making sure the last drop is transferred by touching the two tubes. Discard the glass tube containing the cell debris into a disposal box for glass sharps.

(4) Using another glass bottle-style pipette, transfer 1.0 mL of 1.5 M HCl to the samples and standards. Mix for 10 sec. using a vortex.

(5) Centrifuge the glass tubes for 1 min. at 1000 × *g*. Two distinct liquid phases, EtOAc-HAc (brown) and HCl (clear), should be clearly visible. Clean the surface of each tube with a lab tissue to remove any dust, grease or fingerprints. This tube will function as the sample cell when inserted into the cell holder of the spectrofluorometer.

C. Method Performance Parameters

Within-Run Precision (for EP Lab Staff)

Tech ID	Date	EP µg/dL	SD	%RSD
CG	9/10/99	91	1.3	1.4
CG	5/15/98	62	1.3	2.1
CG	3/12/97	83	2.3	2.8
KM	11/16/01	48	1.9	4.0
KM	10/11/01	42	0.7	1.7
KM	9/18/01	150	3.5	2.3
MFV	11/9/99	65	1.8	2.8
MFV	9/15/99	102	2.3	2.2
MFV	7/15/99	50	0.8	1.6
SS	11/15/99	76	1.1	1.5
SS	5/23/01	70	1.3	1.9
SS	11/3/00	52	0.9	1.8

Accuracy and Precision (based on analyses of NYS RMs)

RM ID	Tech ID	EP target µg/dL	EP found µg/dL	Mean w/in-run SD	Between-run SD	%RSD
Lot 046	SMS	35	34.8	1.1	1.1	3.16%
Lot 046	KAM	35	33.8	1.1	1.7	5.03%
Lot 049	SMS	54	55.3	1.7	1.4	2.53%
Lot 049	KAM	54	53.9	1.4	1.7	3.15%
Lot 045	SMS	116	121.1	3.4	3.9	3.22%
Lot 045	KAM	116	120.0	3.0	3.5	2.92%
Lot 051	SMS	183	176.2	5.9	5.2	2.95%
Lot 051	KAM	183	174.6	4.1	5.9	3.38%

D. Critical Parameters and Troubleshooting

The absorbance measurement of PPIX standard solutions should result in a single band with λ_{max} at 407 nm. Quantitative results for EP quality control materials should within ±6 µg/dL of the target value, for concentrations <40 µg/dL, or within ±15% of the target for values ≥40 µg/dL. These criteria are currently the same as those expected of participants in several US proficiency testing programs for EP.

E. Recording of Data

Plot the instrumental response, i.e., relative fluorescence intensity, for each calibration standard (y) versus the exact PPIX concentration (x), corrected using the stock solution absorbance as described in step 3. Compute the line of best fit by a least-squares linear regression method. From the equation of the line, $y = mx + b$, calculate the PPIX concentration in diluted blood. The value computed from the equation of the line is multiplied by 5.2, a factor which accounts for (a) the extraction efficiency, which in whole blood is 96% or 1.04, and (b) the 1 + 4 dilution of blood with water.

NOTE: there are conflicting views on the efficiency of extracting porphyrins from whole blood samples. Unpublished data from our lab based on 15 diluted blood samples repeatedly extracted with 3 successive aliquots of EtOAc-HAc showed that 96 ±1% of the total extractable protoporphyrin was recovered in the first extraction. Thus, we can assume that the extraction efficiency is 96% providing (i) that all extractable protoporphyrin was removed in the 3 successive extractions, and (ii) the distribution of protoporphyrin IX between the aqueous and organic phase is the same for blood extracts and the aqueous PPIX standards. At the present time, there are no reports of any residual protoporphyrin in the extracted cell debris.

F. Calculations

For EP results reported as part of the NHANES proposal, multiply all data by 0.811 to obtain values standardized to $m\varepsilon = 297$ L mmol^{-1} cm^{-1}.

9. REPORTABLE RANGE OF RESULTS

The detection limit is a function of sensitivity and precision. Theoretically, the method detection limit is 2–3 µg/dL based on 3 SD. In practice, this is never really observed since RBC protoporphyrin is always present and detectable in human blood. The upper limit of the calibration curve is equivalent to around 200 µg/dL.

Values above 200 are found in the human population and are easily measured by performing a dilution of an aliquot from the HCl phase, where PPIX is located.

The top standard, S_t, is equivalent to a blood sample with an EP concentration ~200 µg/100 mL. If any samples, or control materials, have fluorescence intensities above the top standard, additional dilution will be necessary. To dilute further a sample with an EP concentration >S_t, transfer a 500-µL aliquot from the clear aqueous HCl phase of the final extract into a clean glass culture tube. Add 2 mL of 1.5 M HCl, vortex for 3 sec. and measure the reduced fluorescence intensity. The EP concentration must be multiplied further by a factor of 5 to correct for the additional extract dilution with HCL.

10. QUALITY CONTROL (QC) PROCEDURES

New York State blood-based reference materials, certified for EP content in interlaboratory studies, are used daily in this analysis. These materials are prepared from whole blood obtained from lead-dosed animals, preserved with K_2EDTA and lyophilized for long term storage. A minimum of three levels (low, <35, medium 50–100, and high, >150) are used in every analytical run (50 patient samples) in accordance with NYS regulations for this analysis). At least six levels are used for periodic calibration verification, a process required every 6 months under CLIA‘88. Controls are run after the daily calibration and with each tray of 20 patient samples. Since target values are fixed for each reference material, acceptable ranges are fixed based on PT criteria for acceptable results. In this way, each run can be considered a calibration verification. Since each QC material is independently certified in interlaboratory studies, accuracy is assured on a daily basis thus avoiding bias problems.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

In order for patient results to be reported, they must have been part of a group that included a low (normal), medium (abnormal), and high (abnormal) reference material (control) that was reported within acceptable limits.

If any control is outside acceptable range, it is necessary to recalibrate, run controls and re-run all previous patient samples that were within the out-of-control group, again running controls before and after the patient samples.

If, due to instrument failure, reportable results cannot be obtained, the samples must be reanalyzed on the following day.

All samples, controls, and standards are set up in duplicate and the two results for each are considered replicates. Mean replicate results for EP are acceptable if (a) the difference between the two replicate values are < 6 µg/dL for mean EP values of < 40 µg/dL or (b) the difference of the two replicate values are <15% of the mean for mean EP values of ≥ 40 µg/dL.

Analyze specimen a second time if EP ≥ 35 µg/dL or the difference between duplicates is >6 for EP’s <35.

Analyze a third time if (a) the difference between result 1 and result 2 is more than 15% of mean for mean values of 35–100 µg/dL or (b) the mean EP results are >100 µg/dL.

The following EP results should be flagged for suspected poor precision: Results < 35 µg/dL, with an SD ≥ 2; Results 36–99 µg/dL, with an SD ≥ 5; Results >100 µg/dL, with an SD ≥ 10. In such cases, consult the supervisor or lab director.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The most frequent source of problems with this analysis can be traced to the quality of the ethyl acetate supplies used for the extraction reagent (Doran and Mitchell, 1984). Impurities in this reagent can cause fluorescence quenching which leads to erroneously low results. The KI test for impurities (step e) is generally reliable in this respect but results can be misleading because of the subjective nature of the color intensity. If the quality of the ethyl acetate is suspect and the KI test is inconclusive or, perhaps weakly positive, then consider either re-distilling the ethyl acetate or obtaining an alternative supply.

The protection of blood samples and reagents from exposure to bright lights, particularly sunlight or fluorescent tube light is highly desirable since porphyrins are susceptible to photoxidation, which can result in erroneously low values. Blood samples are easily protected by wrapping them in aluminum foil. Avoid using plastic microcollection devices with protective “filters” for bilirubin measurements. These filters do not protect blood samples for the EP assay. In the laboratory, using low-cost photographic filters, which fit over standard fluorescent tubes, and reduced UV radiation are a convenient way of reducing photoxidation of samples and reagents. Extracted blood samples and standard solution should be stored in a light-tight box while the spectrofluorometer is calibrated.

The use of plasticware in the laboratory should be avoided, since organic reagents may leach out impurities that can interfere in the analysis. This may require some modifications to ensure all-glass pipet bottle are used. The key to success is ensuring that all laboratory ware is thoroughly clean and well dried before use. Access to reliable quality control materials is essential to assuring that clinical results are reliable. The availability of such materials is becoming more difficult as the test becomes less widely used. Thus, the onus is on the laboratory performing the test to develop such materials. The operators of the PT programs for EP may be able to help in this respect. One alternative is to prepare QC materials in-house and validate them through interlaboratory studies.

The limited stability of PPIX standards in acid solution (a matter of hours rather than days) requires some attention to timing. For example, it is unwise to perform sample extractions before the instrumentation has been set up, calibrated and optimized. If at all possible, the sample and standard extractions, and the fluorometric readings should be carried out in a single operation and not separated by a long interval, e.g., a lunch break. The entire procedure, including reporting results, can be easily accomplished in typical 8-hr shift if no problems are encountered.

A. Identifying and resolving problems with ethyl acetate quality

Some commercial supplies of ethyl acetate contain impurities such as oxidizing agents that can quench PPIX fluorescence thus reducing relative fluorescent intensity. The problem almost always appears as low or inconsistent results for internal or external QC samples. Some problems can be traced to the use of plastic laboratory ware. Store all EtOAc solutions in glass bottles rather than plastic, since EtOAc can leach materials which will quench fluorescence. Using clean, acid-washed, and well-dried glass apparatus for all reagents and procedures will eliminate many problems. If the problem can not be traced to plastic ware, then the quality of the ethyl acetate used for the extraction is suspect. It is preferable to purchase bottles containing 2 liters or less since EtOAc left exposed to air may become oxidized thus producing quenching agents. One test that can indicate the potential for a problem with quenching agents in EtOAc is the potassium iodide test for impurities (Support Protocol 1). If the KI test is positive, the ethyl acetate might have to be re-distilled from glass in the laboratory (Support Protocol 2).

B. Support Protocol 1 Testing ethyl acetate stock for quenching impurities

(1) Equipment:

(a) 150 mL glass beaker

(b) 10% (w/v) potassium iodide solution

(c) 100 mL glass beaker

(d) 100 mL graduated glass cylinder

(2) Procedure:

(a) Transfer 50 mL of ethyl acetate into a 100-mL beaker, and place it on a white surface, e.g., paper.

(b) Add 10 mL of 10% KI solution to the ethyl acetate and swirl to mix.

A distinct yellow color, due to the oxidation of iodide, indicates that the ethyl acetate stock contains impurities that will quench PPIX fluorescence. Avoid using any ethyl acetate batch that yields a positive KI test. Use only supplies that do not produce a yellow color upon addition of 10% KI. If you cannot locate a suitable clean supply of ethyl acetate, the material can be cleaned up as described in Support Protocol 2.

C. Support Protocol 2 Procedure for ethyl acetate clean-up by glass distillation.

(1) Equipment:

(a) 2-L round-bottom glass boiling flask $24/40.

(b) ~15 inch glass fractionation column with $24/40 ground-glass joints, wrapped in insulation and aluminum foil.

(c) ~15 inch glass condenser with elbow to connect with a collection vessel ($24/40).

(d) 2-L glass bottle with size $24/40 ground glass joint opening.

(e) 2 ring stands and a lab jack.

(f) Rheostat, e.g., 0-120 V output, 1500 W Glas-Col, or equivalent.

(g) 500 W heating mantle, e.g., Glas-Col or equivalent.

(h) Teflon boiling stones.

Procedure:

CAUTION: This is a hazardous procedure and must be done under a laboratory hood. Remember that ethyl acetate and its vapors are highly flammable. When a bad batch of ethyl acetate has been identified, it will be necessary to wash out all pipet bottles with a clean batch to prevent cross contamination.

(a) Pour 1.5 L of commercial ethyl acetate into the 2-L round-bottomed flask and add a few Teflon boiling stones.

(b) Connect the flask to the column and condenser arrangement. Turn on the water flow to the condenser.

(c) Set the rheostat to arbitrary setting 75 until the ethyl acetate is almost boiling. Decrease heating to arbitrary setting 65. Condensed ethyl acetate should be produced steadily as rapid but distinct drops.

(d) Discard the first 100–200 mL of condensate. Use to rinse the collection/storage bottles.

(e) Collect about 1.0–1.2 L of condensed ethyl acetate and transfer to clean, amber bottle. The procedure may be repeated one more time without emptying the round-bottomed flask.

NOTE: Always check the ethyl acetate reagent just before use with the KI test. Don’t allow the flask to boil dry; this is not only hazardous but will require that you start again! To avoid this problem: (a) use a timer; (b) decrease the heating rate further yet upon collecting ~800 mL. Don't heat the flask too rapidly at the start or ethyl acetate will be directly ejected up into the fractionating column.

D. Reagents and Solutions

Use NCCLS Type I deionized water such as Millipore Milli-Q or equivalent for the preparation of all solutions.

(1) Ethyl acetate (EtOAc), acetic acid (HAc) mixture, 4 + 1 by volume.

Pour 800 mL EtOAc into a 1-L graduated cylinder. Add 200 mL acetic acid. Stir well with a clean glass rod and pour into a 1-L glass bottle-type pipet. Check the appropriate MSDS for important information on this solvent.

(2) Hydrochloric acid, 1.5 M

Pour 500 mL of deionized water into a 1-L class A volumetric flask. Carefully add 125 mL of concentrated HCl using a graduated cylinder. Dilute to volume with deionized water, mix and transfer to a bottle-type dispenser. Check the appropriate MSDS for important information on this acid.

(3) Potassium iodide, 10 % (w/v)

Dissolve 10 g of potassium iodide in 100 mL of deionized water. Use only for the KI test for ethyl acetate and discard immediately after use. Check the appropriate MSDS for important information on this chemical.

13. REFERENCE RANGES (NORMAL VALUES)

A normal value is <35 µg/dL (NCCLS, 2001).

Normal clinical test results for EP vary slightly comparing men to women and children, based upon data derived from the NHANES II studies (Yip et al., 1984). However, the NHANES II data were calculated with an mε value of 297, and a correction must be made to convert those data into equivalent EP results measured using the 241 value. Taking the 95th percentile NHANES data for men, women, and children, after excluding those with iron-deficiency, anemia, and elevated blood lead levels (National Committee for Clinical Laboratory Standards., 1996), yields rounded upper threshold values of 35 µg/dL, 37 µg/dL and 38 µg/dL whole blood, respectively. Note that these values are close to the most commonly accepted upper threshold value for EP, 35 µg/dL whole blood, recommended by the CDC in their 1985 document for screening children exposed to lead .

14. CRITICAL CALL RESULTS (PANIC VALUES)

A critical call results is ≥100 µg/dL

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Samples are stored frozen at –70°C until they are analyzed. The samples are brought to room temperature on the morning of the analysis. The residual samples are replaced in the racks and refrozen.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

Generally, if a problem with the method exists, samples are held in the freezer until it can be resolved.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

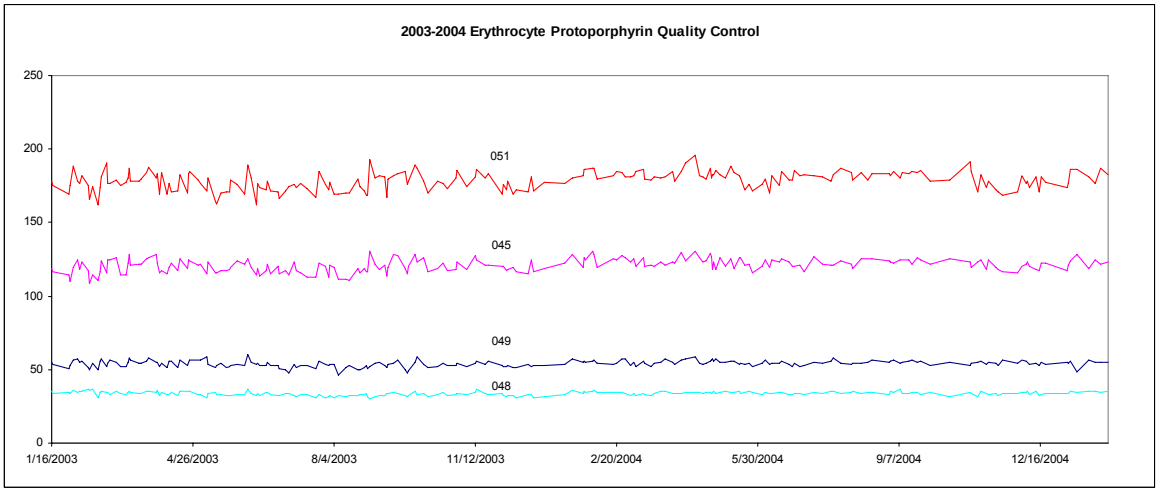
18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Residual sample from these assays is maintained in the same freezer that is used to hold samples waiting to be processed.

19. Summary Statistics and QC Graphs

A. Erythrocyte Protoporphyrin

Summary Statistics for Erythrocyte Protoporphyrin by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
048	216	1/16/2003	2/2/2005	33.7	1.3	3.8
049	217	1/16/2003	2/2/2005	54.0	2.2	4.0
045	217	1/16/2003	2/2/2005	121.0	4.3	3.6
051	217	1/16/2003	2/2/2005	178.5	6.2	3.5



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