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: *Nutritional Biochemistry Branch*
Division of Laboratory Sciences
National Center for Environmental Health, CDC

Contact: *Dr. Eric J. Sampson, Director*
Division of Laboratory Sciences

Important Information for Users

CDC 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 1999–2000 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label (and SI units)
lab06	LBXEPP	Erythrocyte protoporphyrin

1. Summary of Test Principle and Clinical Relevance

   Free erythrocyte protoporphyrin (FEP) is measured by a modification of the method of Sassa et al. (1). Protoporphyrin is extracted from EDTA-whole blood into a 2:1 (v/v) mixture of ethyl acetate-acetic acid, then back-extracted into diluted hydrochloric acid. The protoporphyrin in the aqueous phase is measured fluorometrically at excitation and emission wavelengths of 404 and 658 nm, respectively. Calculations are based on a processed protoporphyrin IX (free acid) standard curve. After a correction for the individual hematocrit is made, the final concentration of protoporphyrin in a specimen is expressed as micrograms per deciliter of packed red blood cells (µg/dL RBC).

2. Safety Precautions

   This method is performed under an exhaust hood (not a laminar-flow hood) because the ethyl acetate-acetic acid and hydrochloric acid fumes are very irritating. Observe Universal Precautions. Wear gloves, lab coat, and safety glasses at all times. Treat all specimens as potentially positive for HIV and Hepatitis B. Dispose of leftover acid solutions as hazardous wastes. All leftover blood specimens and materials that have been in contact with blood must be autoclaved before disposal.

   Reagents and solvents used in this study include those listed in Section 6. Material safety data sheets (MSDS) for these chemicals are readily accessible as hard copies in the lab. If needed, MSDS for other chemicals can be viewed at <http://www.ilpi.com/msds/index.html> or at <http://intranet.cdc.gov/ohs>.

3. Computerization; Data System Management

   a. After a run is complete and any additional corrections by the analyst are made, the result file (containing the patient data as well as the QC data) is electronically transferred to the appropriate analyte-specific subfolder in Q:/ITN/Nutrition Lab/Import into Access on the NCEH/DLS Local Area Network (LAN). The analyst also gives a hardcopy of the result file to the reviewing supervisor. After the reviewing supervisor approves the final values for release by checking off the bench and blind QC values and signing the hardcopy, he/she sends an email to the computer support staff that the data has been released to be imported into the NHANES 1999+ database that is located in Microsoft Access; the computer support staff imports the data into the NHANES 1999+ database by using a macro. Data entry is verified by the computer support staff and the supervisor. Data is transmitted electronically several times weekly to Westat's ISIS computer system, and transferred from there to NCHS. Abnormal values are confirmed, and codes for missing data are entered by the analyst and are transmitted as part of the data file to the Westat ISIS computer, and are eventually forwarded to NCHS. Westat also prepares the abnormal report notifications for the NCHS Survey Physician.

   b. Files stored on the network or CDC mainframe are automatically backed up nightly by DLS LAN support staff and CDC Data Center staff, respectively. Backup of the daily data containing all raw data files and result files for each run are the responsibility of the analyst. Typically these files are backed up once a week onto a floppy disk or a CD-ROM using a CD writer.

   c. Documentation for data system maintenance is contained in printed copies of data records, as well as in "system log" files on the local hard drives used for the archival of data.

4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

   a. No special dietary instructions are given to donors.

   b. Specimens for erythrocyte protoporphyrin analysis should be fresh or frozen EDTA-whole blood. Heparinized blood may be used, but it is not preferred because of the tendency of the blood to form microclots upon prolonged storage. If possible, hematocrit data should be collected in order to correct for the effects of anemia, and the final FEP concentration should be reported as µg/dL RBC.

   c. The optimal amount of sample is 1 mL; the minimum is about 100 µL.

d. Specimens may be stored in glass or in plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

e. Protoporphyrin is stable for years at –20°C and below. Quality control pools for the HANES Lab are normally stored at –70°C for maximum stability. Several freeze-thaw cycles appear to have minimal effect on the specimen. However, after prolonged storage at 4–8°C, blood specimens undergo necrosis, which results in formation of fluorescent compounds that can interfere with FEP analysis.

f. Specimens should generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site where the blood was drawn.

g. Samples that are partially clotted may not give accurate test results. In addition, care should be taken not to introduce any fluorescent artifacts into the sample during processing through the use of equipment such as wooden applicator sticks.

h. Specimen handling conditions are outlined in the Policies and Procedures Manual of DLS (copies are available in the Nutritional Laboratory and the electronic copy of this file is located at Q:/ITN/Nutrition Laboratory/CLIA). The protocol discusses collection and transport of specimens and the special equipment required. In general, serum should be transported and stored at no more than –20°C. Samples thawed and refrozen less than five times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood or plasma should be transferred into a sterile Nalge cryovial labeled with the participant's ID.

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable for this procedure

6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

a. Reagent Preparation

1) 7.0 mol/L hydrochloric acid (HCl) (for hydrolysis)

Dilute 551 mL concentrated HCl to volume with deionized water in a 1-L volumetric flask.

2) 1.62 mol/L HCl (for weekly absorbance readings)

Dilute 128 mL concentrated HCl to volume with deionized water in a 1-L volumetric flask.

3) 0.43 mol/L HCl (for analysis extraction)

Dilute 68 mL concentrated HCl to volume with deionized water in a 2-L volumetric flask.

4) 1.5 mol/L HCl (for blanking spectrophotometer)

Dilute 118 mL concentrated HCl to volume with deionized water in a 1-L volumetric flask.

These dilutions assume concentrated HCl to be 12.7 mol/L in the lot of HCl used. The molar concentration of different lots of HCl should be calculated by using the following formula:

$$\text{mol/L} = \frac{\text{relative density} \times \% \text{ HCl}}{36.453}$$

5) 2:1 (v/v) ethyl acetate acetic acid

Working under a hood, combine 200 mL of ethyl acetate and 100 mL of glacial acetic acid. Mix the solution well; this volume is sufficient for the standards, controls, and 80 specimens in duplicate. (Prepare the reagent daily, immediately before sampling the whole blood.)

> Ethyl acetate quality appears to be the most frequent source of problems in performing the assay. The grade of the reagent is less critical than its ability to pass the potassium iodide (KI) test for quenching agents. If a quenching problem arises, test the ethyl acetate for the presence of impurities such as peroxides. Transfer 50 mL of ethyl acetate into a glass beaker and place the beaker on a white surface (e.g., paper). Add 10 mL of 10% (w/v) KI in deionized water solution and gently swirl the contents of the beaker. The presence of a distinct yellow color indicates impurities that would result in low, out-of-control values for quality control material. Reject any bottles of ethyl acetate that test positive. Purchase 500-mL size bottles; larger bottles may pass the KI test initially, but the contents may degrade as the bottles are repeatedly opened for use.

b. Standards Preparation

Prepare all standard solutions in actinic glass volumetric flasks, in very reduced light. At present there are no NIST SRM's available for FEP standardization. The standard material used for the HANES method contains the highest purity standard material available, and that purity is confirmed by TLC, HPLC, fluorescence, and spectroscopy.

(1) Protoporphyrin IX standards
Concentrations are expressed in terms of protoporphyrin I× free acid. The millimolar absorptivity of protoporphyrin IX conventionally has been determined in 1.5 mol/L HCl; thus, the weekly absorbance reading of the hydrolysate is determined at this acid concentration (2).

(2) 200 mg/L protoporphyrin IX free acid hydrolysate (stock standard)
Weigh 42.0 mg protoporphyrin IX dimethyl ester (PPIX DME). Dilute to volume in a 200-mL actinic volumetric flask with 7 mol/L HCl, washing PPIX off the weighing paper with a few drops of formic acid. Add a small stirring bar, cover the flask with aluminum foil, and mix the contents at 2–0-25°C for 3 hours, using a magnetic stirrer. (Prepare when the absorbance of the standard falls below 0.5100.)(3)

(3) 10 mg/L intermediate stock
After 3 hours, dilute 25.0 mL of 200-mg/L solution with deionized water to volume in a 500-mL actinic volumetric flask, to yield a 10-mg/L solution in 0.35 mol/L HCl. This stock solution will be used to prepare the working standards for daily instrument calibration. (Store in actinic bottles at 4–8°C. Allow the solution to reach consistent room temperature before using.)

(4) 1 mg/L standard for weekly absorbance readings
Dilute 5.0 mL of 10-mg/L intermediate stock (brought to ambient temperature before dilution) to volume in a 50-mL actinic volumetric flask with 1.62 mol/L HCl to yield a 1-mg/L protoporphyrin IX standard in 1.5 mol/L HCl. Use an aliquot of this standard for absorbance readings.

The theoretical concentration of this solution with respect to protoporphyrin IX free acid (PPIX FA) is calculated as follows:

$$\frac{42 \text{ mg PPIX DME}}{200 \text{ mL}} \times \frac{562.27 \text{ mg PPIX FA}}{590.72 \text{ mg PPIX DME}} = 0.1999 \text{ mg PPIX FA/mL}$$

$$\frac{0.1999 \text{ mg PPIX FA}}{\text{mL}} \times \frac{25 \text{ mL}}{500 \text{ mL}} \times \frac{10 \text{ mL}}{100 \text{ mL}} = 0.0009995 \text{ mg/mL PPIX FA (or 99.95 µg/dL)}$$

$$\frac{99.95 \text{ µg}}{1 \text{ dL}} \times \frac{1 \text{ mmol}}{562.27} \times \frac{10 \text{ dL}}{1 \text{ L}} \times \frac{1 \text{ mg}}{1000 \text{ µg}} = 0.00178 \text{ mmol/L PPIX FA}$$

c. Preparation of Quality Control Materials

Two levels of blind QC pools may be prepared from human and lead-spiked bovine blood with elevated or decreased protoporphyrin levels. Pool the whole blood in acid-cleaned glass bottles and mix well on a magnetic stirrer. Using sterile technique under a laminar-flow hood and a Micromedic Digiflex, dispense the blood in 500-µL aliquots into 2.0-mL Nalge cryovials. Cap and label the vials with NHANES bar-coded labels that have been specially prepared for the QC pools. Store the pools at –70°C at the CDC Serum Bank in Lawrenceville where they will be inserted randomly into the NHANES runs.

Three levels of bench QC pools (low-human, medium-bovine, and elevated-bovine) are prepared in the same manner as above. Store the pools at –70°C until needed.

Select twenty vials of each level at random for characterization of the quality control limits and for testing of homogeneity.

d. Other Materials

(1) Protoporphyrin IX, dimethyl ester, 99.3% purity, grade 1 (Sigma Chemical Co., St. Louis, MO), or 99.9% purity (Porphyrin Products, Logan, UT).

Store 20°C over a desiccant. Purchase of one lot is recommended. If possible, prepare (and label) for storage aliquots of each lot in ampules.

(2) Ethyl acetate, high-performance liquid chromatography (HPLC) grade (J.T. Baker Co., Phillipsburg, NJ).

(3) Acetic acid, glacial, "Baker Analyzed" (J.T. Baker Co.).

(4) Hydrochloric acid (HCl), concentrated, "Baker Analyzed" (J.T. Baker Co.).

(5) Kimble 10- x 75-mm disposable glass culture tubes (Kimble Div., Owens-Illinois Co., Toledo, OH).

(6) Parafilm M (American Can Co., Greenwich, CT).

(7) Actinic glass volumetric flasks (Corning Glassworks, Corning, NY).

Wash all nondisposable glassware used in this assay in 10% (v/v) hydrochloric acid and rinse them six times with deionized water.

(8) Formic acid, 88% (v/v), reagent grade (J.T. Baker Co.).

(9) Deionized water, ≥1.0 MΩ/cm at 25°C (Continental Water Co., Atlanta, GA).

e. Instrumentation

(1) Hitachi model F-2000 fluorescence spectrophotometer, with R928 photomultiplier tube, xenon lamp, and custom-made microcell (10- x 75-mm) holder positioned to allow the passage of light through the aqueous phase only (Hitachi Instruments Inc., Danbury, CT).

(2) Cary model 3E double-beam spectrophotometer (Varian Instrument Group, Sugar Land, TX).

(3) Vortex mixer (Fisher Scientific Co., Fairlawn, NJ).

(4) Mettler model PM400 balance (Mettler Instruments Corp., Hightstown, NJ).

(5) Beckman TJ-6 centrifuge (Beckman Instruments Co.).

(6) Three Digiflex automatic dispensers, (Titertek Instruments Inc., Huntsville, AL) equipped with the following:

(a) A 10-mL dispensing syringe and a 2-mL sampling syringe.
(b) A 2-mL dispensing syringe and a 40-µL sampling syringe.
(c) A 10-mL dispensing syringe.

7. Calibration and Calibration Verification Procedures

a. 0-80 µg/dL Working Standards

(1) A new standard curve is established with each patient run. Using the Digiflex, prepare the following working standards by diluting the 10 mg/L standard with 0.43 mol/L HCl according to the dilution scheme shown in Table 1 (APPENDIX).

> Be sure to work under very subdued lights when diluting and extracting the standard materials, since they are photo-labile.

(2) Extract the standards as described in the procedure section of this method. The Hitachi F-2000 will prompt the analyst to read the nine standards in duplicate, starting with level 0.

(3) When all the standards have been read, the instrument will draw the linear standard curve (x = concentration, y = intensity). Additional statistical information may also be obtained upon request, including the correlation coefficient (r). The correction coefficient squared must be greater than or equal to 0.9960 for a standard curve to be considered valid.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

> To protect hands against acids and solvents during sampling, wear nitrile gloves. To avoid evaporation and degradation of specimens, process samples as rapidly as possible. After centrifugation, samples are stable for 1-2 hours.

a. Preliminaries

(1) Thaw specimens and quality control materials of frozen EDTA-whole blood at room temperature. (Control pools with elevated levels of FEP have been prepared from blood (EDTA-anticoagulated) collected from cows that have been fed lead acetate.)

(2) Once per week, using the spectrophotometer and quartz cuvettes, measure the absorbance at the wavelength maximum (approximately 407-408 nm) of the 1 mg/L standard in 1.5 mol/L HCl standard solution against a blank of 1.5 mol/L HCl, scanning from 380 to 420 nm. This measurement will be used in determining the quality of the standard. Soak cuvettes with 10% HCL after use, and rinse thoroughly with deionized water followed by ethanol to remove water droplets.

(3) Prepare the working standard dilutions from the 10 mg/L standard solution, using 0.43 mol/L HCl as a diluent. These dilutions are unstable; therefore, prepare them as rapidly as possible.

(4) Prepare the 2:1 ethyl acetate-acetic acid mixture, and fill a dispenser bottle of one Digiflex dilutor for delivering 1.0 mL of reagent. Fill the dispenser bottle of another Digiflex dilutor with 0.43 mol/L HCl for delivery of 1.0 mL. (Place dilutors under a hood to minimize exposure to fumes.)

b. Sample preparation

(1) Before sampling, thoroughly vortex each standard dilution, quality control pool, or whole blood specimen. Sample in this order: standards (including the 0 standards to be used as blanks), quality control pools, and whole-blood specimens in duplicate.

(2) Using the first Digiflex set in “sample-dispense” mode, transfer 10 µL of the sample from its container, followed by 1.0 mL of the ethyl acetate-acetic acid mixture, to a 10- x 75-mm disposable glass tube. Prepare each sample/QC pool/standard in duplicate.

(3) Add 1.0 mL of the 0.43 mol/L HCl to each sample using the second Digiflex in “dispense” mode. Wrap each tube with Parafilm, and vortex thoroughly for 10 sec.

(4) When all sampling is completed, centrifuge all tubes for 4 min at 1400 rpm.

c. Hitachi F-2000 Spectrofluorometer Settings:

Set the parameters for the F-2000 as shown in Table 2 (APPENDIX).

d. Operation

(1) Turn on the F-2000 POWER switch. Wait approximately 10 seconds, then press the LAMP START button. (Do not depress this button for longer than 5 seconds) Turn on the MAIN power switch.

(2) Allow at least 30 min for the F-2000 to warm up and stabilize after the xenon lamp has been ignited.

(3) Select 'TEST MENU' from the F-2000 main menu.

(4) Select 'LOAD' followed by the number corresponding to the 'PROTO' method.

(5) Insert each tube into the sample holder, close the compartment door, and press [EX] to read. The tubes should be read starting with the standards, followed by the controls, samples, and a final set of controls. See the Hitachi F-2000 manual for further information on the operation of the instrument.

(6) For samples outside the range of the standard curve, dilute the whole blood with an equal volume of normal saline and extract. Read the sample and multiply the results by a factor of 2 when reporting values. If necessary, a 1:5 dilution with saline may also be used.

(7) Turn off the instrument in reverse order. After the power source has been turned off and the lamp has gone out, turn the switch back to the 'ON' position and allow the fan to cool the lamp for at least 15 minutes.

e. Replacement and Periodic Maintenance of Key Components

(1) Xenon lamp: A spare lamp should be available. Order another if the spare is used for replacement.

(8) Printer tape: A supply of printer tape should be on hand.

(9) Sensitivity Check for the F-2000 spectrofluorometer:

The sensitivity check is designed to measure the ratio of lamp signal intensity to instrument background electronic noise [hence, “S:N”] in Raman scattered light of water under a predetermined measurement condition. This procedure should be performed any time the lamp is changed or if the fluorescent intensity of the calibrating standard drops significantly.

1. After turning on the xenon lamp, allow it to stabilize for at least 30 min.

2. Adjust the xenon lamp to its optimum position (i.e., as reflected by highest signal intensity). Refer to the F-2000 user’s manual for xenon lamp calibration instructions.

3. Set deionized water in the sample compartment. Ensure that the outside of the cuvette has been wiped with alcohol to remove any fluorescent artifacts such as oils from fingerprints.

4. Refer to the F-2000 user’s manual for “System Performance Test” instructions.

5. Approximately 10 minutes later, the following results of the sensitivity check will be displayed: S/N, drift, peak wavelength of Raman spectrum(S), and noise value (N).

6. Print this report and file it in the F2000 instrument log.

f. Calculations

(1) The F-2000 will calculate the calibration curve and the specimen concentrations. The correlation coefficient squared should be 0.9960 or better, and the slope should be between 0.9000 and 1.1000.

(2) The millimolar absorptivity of protoporphyrin IX free acid in 1.5 mol/L HCl has been determined in our laboratory to be 297 ± 1 (600 observations from 1976 to 1988) (4-6). The purity of our material has been confirmed by elemental analysis and high-performance liquid chromatography of the extracted protoporphyrin IX free acid. Calculate the actual concentration of the 1 mg/L (0.00178 mmol/L) working standard using the following equation:

$A = \varepsilon bc$

Where:

A = absorbance reading
ε = 297, the millimolar absorptivity of protoporphyrin IX free acid in 1.5 mol/L HCl
b = cuvette path length, 1 cm
c = concentration, in mmol/L

For example, if the daily absorbance reading of the 1 mg/L standard at wavelength maximum is 0.520, then:

$$c = \frac{0.520}{(297\ \text{L/mmol\_cm})\ (1\text{cm})} = 0.00175\ \text{mmol/L}$$

Then:

(.00175 mmol/L)(562.27 mg/mmol)(1000 µg/mg)(1L/10dL) = 0.9840 mg/L PPIX FA

Consider 0.9840 as a percentage of 100 µg/dL (1 mg/L), and correct the standard curve accordingly:

10 µg/dL × 0.9840 = 9.84
20 µg/dL × 0.9840 = 19.68 etc.

Perform a linear regression, with x equal to the corrected standard concentration and y equal to the fluorescent intensity reading. Using the slope of the standard curve and assuming zero intercept, calculate the concentration of protoporphyrin IX per deciliter of whole blood for each specimen.

When using the Hitachi F-2000, you need not perform these calculations. The instrument will create a calibration curve using the standards and calculate the concentration of the unknowns automatically. Results on the instrument printout are given in µg/dL.

To correct for hematocrit and express results as µg/dL of RBC, use this formula:

$$\frac{\text{µg/dL whole blood} \times 100}{\text{hematocrit}} = \text{µg/dL RBC}$$

(3) Repeat a specimen analysis when duplicate values do not check within 10%, when any sample has a calculated value of 100 µg/dL RBC or more (i.e., higher than the 99th percentile of the NHANES III data distribution for EP), or if the µg/dL value of the sample exceeds 80 µg/dL (i.e., the highest standard). The whole blood sample should be diluted 1:2 (i.e., 1+1, or at a higher dilution if appropriate) with 0.9 g/dL saline and re-extracted. The results should be multiplied by the dilution factor before they are reported.

g. Special Method Notes

Once the standards have been extracted, continue through the analysis without interruption to minimize error due to incomplete extraction. Some small variations in fluorescence values may be due to poor quality 10- × 75-mm tubes used as cuvettes. Blood specimens are very stable;

standards are somewhat labile and must be processed under reduced light. Accurately weighing the PPIX DME is critical, as is avoiding prolonged hydrolysis time (i.e., >3 hours).
If hematocrit correction is not used for reporting data, the traditional cutoff level is 35 µg/dL to indicate a possible blood lead level greater than 25 µg/dL.

Although we have not found this practice necessary (because our samples are received frozen for NHANES), both New York State and the Wisconsin State Laboratory of Hygiene recommend a 1:5 dilution of fresh whole blood with saline in microtiter plates to ensure complete lysis of cells. Both also use a concomitant dilution of standard concentration.

9. Reportable Range of Results

Protoporphyrin results are reportable undiluted when the values are less than 80 µg/dL. Whole blood samples with concentrations of 80 µg/dL or greater should be diluted at least 1:2 with 0.9 g/dL saline and reanalyzed, with the results multiplied by the dilution factor.

10. Quality Control (QC) Procedures

FEP is measured by a "batch" method (i.e., all specimens, standards, and QC pools simultaneously undergo the same processes, such as extraction). On an average day, 80 specimens are analyzed in duplicate, with 3 levels of bench QC (low-human, medium-bovine, and elevated-bovine) analyzed at the beginning and end of each run.

In every batch of 20 specimens analyzed, either one low- or one high-concentration blind quality control pool is randomly inserted. The blind pools are aliquoted and labeled in exactly the same fashion as the NHANES 1999+ specimens and are inserted in the specimen batches by the Serum Bank personnel when the specimens are received from the field and racked for analysis.

The results from the pools are checked after each run. The system is declared “in control” if all three QC results are within 2s limits and the run is accepted. If one of the three QC results is outside the 2s limits then apply rules below and reject if any condition is met - the run is then declared “out of control”:

- 1_{3s} Any of the three QC results are outside the 3s limit
- 2_{2s} Two of the three QC results in the run are outside the 2s limit (same side of mean)
- R_{4s} Sequential QC results (either within the run or across runs) are outside the 2s limit on the opposite sides of the mean
- 10_x Ten sequential QC results (across pools and across runs) are on the same side of the mean

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared “out of control” for that analyte as assessed by internal (bench) QC.
The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated quarterly. When necessary, limits are updated to include more runs.

While a study is in progress, electronic copies of the QC results from each run are stored in the analyte-specific folder on Q:/ITN/Nutrition Lab/Data handling/Import into Access. Electronic copies of the tracking of the QC results over time are stored in the analyte-specific folder on Q:/ITN/Nutrition Lab/Data handling/QC Results in Excel. The analyst also keeps a hardcopy of the QC results from each run.

Blind QC results are examined by using similar criteria. The supervisor also evaluates the slope, intercept, and R_2 values for trends. The overall coefficient of variation for this method has been 4-5% over the entire analytical range.

11. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

    If one or more quality control samples fall outside the 3SD limits, take the following steps:

    a. Prepare fresh calibration standards, and repeat the entire curve using the freshly prepared standards.

    b. Prepare fresh dilutions of all whole-blood quality-control samples and re-analyze them.

    c. Perform System Performance Test on F-2000. Adjust light source if necessary.

    If the steps outlined above do not result in the correction of the "out of control" values for QC materials, the supervisor should be consulted for other corrective actions. No analytical results should be reported for runs not in statistical control.

12. Limitations of Method; Interfering Substances and Conditions

    a. The range of linearity for the method is equivalent to the 0–80 µg/dL standards range; because individual final results are dependent on hematocrit. It is not possible to stipulate an upper end of linearity based on a final calculated answer in µg/dL RBC. Instead, any sample with a raw concentration in fluorescence units greater than that of the 80 µg/dL is reanalyzed as a diluted sample. Limits of detection are based on measuring 10–20 replicates of the 0 standard as unknowns and calculating a mean + 3 standard deviations as the LOD.

    b. Interfering substances

        (1) External fluorescent contamination may result from the sample coming in contact with items such as wooden applicator sticks or glassware that has residual soap from washing. Some hand lotions may also contribute extraneous fluorescence contamination on cuvettes when they are handled.

        (2) Concentrated acid tends to quench fluorescence. Rinse all glassware thoroughly after it has been rinsed with acid.

13. Reference Ranges (Normal Values)

    The following ranges for FEP (7) are used in evaluating data:

    Females tend to have higher average values than males; children tend to have higher average values than adults.

    Children <6 years old : 36–97 µg/dL RBC
    Children 6–14 years old : 37–83 µg/dL RBC
    Males 15–74 years old: 33–81 µg/dL RBC
    Females 15–75 years old: 37–93 µg/dL RBC

14. Critical Call Results (“Panic Values”)

    Since data is transmitted to Westat several times weekly, all abnormal values are transmitted immediately to the NCHS Survey Physician for follow up. Any value of an NHANES 1999+ sample 100 µg/dL RBC requires follow up. (Previously 90 µg/dL RBC was used as a cutoff, but this value is within the 95th percentile of data for several age groups, although it usually does indicate that proportion of those age/sex groups that are more subject to iron deficiency.) For all other non-NHANES studies, elevated values are faxed to the principal investigator, who is responsible for follow up.

15. Specimen Storage and Handling During Testing

Specimens should remain at room temperature during testing. Special care must be taken to keep samples out of direct light.

16. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

There are no acceptable alternative methods for performing this test. In case of system failure, store all whole blood specimens at –20°C until the system is functioning.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)

Test results are reported to the collaborating agency at a frequency and by a method determined by the study coordinator. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as an ASCII text file or Excel file, either through electronic mail or on a diskette.

For NHANES 1999+, all data are reported electronically several times weekly to the Westat ISIS computer and then are transferred to NCHS. For some smaller studies, hard copies of a data report are sent, as well as the results in electronic format.

Elevated values are reported as stated in 14.

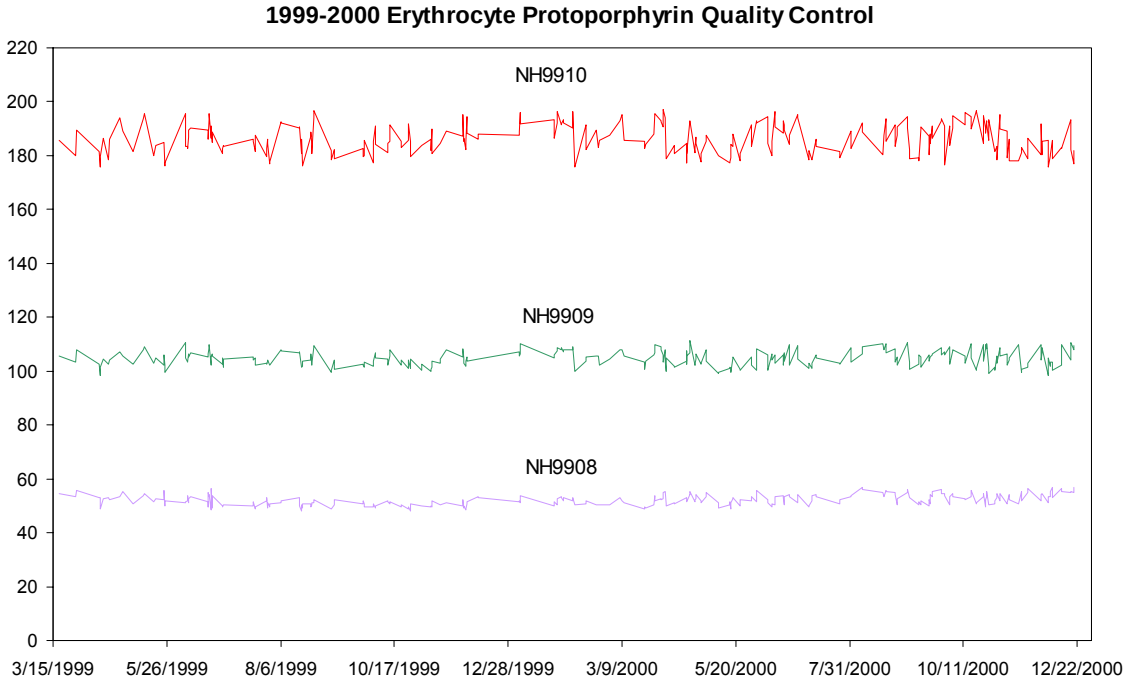
18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

The Microsoft Access database is used to keep records and track specimens for NHANES 1999+. If erythrocyte protoporphyrin analyses are used for smaller, non-NHANES studies, records are kept on files in Q:\ITN\Nutrition Lab on the DLS LAN. We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the NHANES study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual blood from these analyses for non-NHANES studies may be discarded at the request of the principal investigator, or may be transferred to the CDC CASPIR facility for use by other investigators. Very little residual blood will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

19. Summary Statistics and QC Graphs

Table 1. Summary Statistics for Erythrocyte Protoporphyrin by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
NH9908	275	3/19/1999	12/20/2000	52.07	2.02	3.87
NH9909	273	3/19/1999	12/20/2000	104.68	2.93	2.80
NH9910	273	3/19/1999	12/20/2000	186.14	5.57	2.99



Appendix (Tables 1-2)

Table 1. Preparation of working standards

Working Standard Concentration (µg/dL)	Volume 10 mg/L Standard (µL)	Volume 0.43 mol/L HCl (µL)	Final Volume (µL)
80	400	4600	5000
70	350	4650	5000
60	300	4700	5000
50	250	4750	5000
40	200	4800	5000
30	150	4850	5000
20	100	4900	5000
10	50	4950	5000
0	0	5000	5000

Table 2. Hitachi F-2000 spectrofluorometer settings

Parameter	Setting
Data Mode	Conc
num WL	1
Sample Num	1
WL 1 (nm)	EX 404, EM 658
Replicates	2
Init Delay (sec)	0
Integ Time (sec)	5
Hi Limit	40
Lo Limit	0
Unit Label	Other
Curve Type	1st order
Num Stds	9
Curve Mode	New
Response (sec)	0.1
Bandpass [slitwidth] (nm)	EX 10 EM 10
PM Voltage (V)	700
Text Print	On