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Erythrocyte Protoporphyrin Testing; Approved Guideline

This document contains recommendations for the measurement, reporting, and interpretation of erythrocyte protoporphyrin using hematofluorometric and extraction measurement methods.

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Erythrocyte Protoporphyrin Testing; Approved Guideline

Abstract

Erythrocyte Protoporphyrin Testing; *Approved Guideline* (NCCLS document C42-A) is a comprehensive document for use by laboratorians who perform erythrocyte protoporphyrin (EP) determinations; its aim is to reduce/eliminate the lack of uniformity in current measurement practices. The biochemistry and pathology of EP are discussed, the history of EP determinations is summarized, and the applications of the test are defined. The document recommends the adoption of a specific molar absorptivity constant for the standardization of EP methods and the universal adoption of reporting units expressed as the molar ratio of protoporphyrin to heme. Detailed methods for the measurement of EP by extraction and hematofluorometry are included, and the interpretation of EP results is discussed.

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Contents

Foreword

In the past twenty years, there has been tremendous change in the application and analytic methodology of erythrocyte protoporphyrin (EP) testing. Before the early 1970s, testing for EP was generally restricted to research settings. The discovery of an association between EP and blood lead created a substantial demand for the test and, in 1978, the Centers for Disease Control (now the Centers for Disease Control and Prevention) recommended EP as the primary screening test for childhood lead poisoning. It was also learned that EP is a sensitive indicator of iron status, which provides another useful application for the EP test.

This interest in EP led to the development of assay methods suitable for the clinical laboratory: first a two-step extraction procedure followed by conventional fluorometry, and later the introduction of compact, dedicated front-face fluorometers (hematofluorometers), which allowed an immediate, simple, and inexpensive test result using a drop of whole blood.

However, these analytic methods are not without problems. When hematofluorometers (HF) were introduced, primary standards did not exist, and the available secondary standards proved to be unreliable. Generally, the standardization of HF is traceable to extraction results, but discrepancies between these methods are well documented in the literature. In fact, the standardization of extraction methods has been contentious, due, in large part, to the lack of stability of protoporphyrin in solution and a lack of consensus as to the molar absorptivity of the molecule. In addition, a lack of uniformity in reporting units exists, with results reported either as some ratio of protoporphyrin to hemoglobin or as a simple weight per whole blood volume concentration.

This document addresses these problems. It provides recommendations for uniform procedures, recommends the universal adoption of molar ratio reporting units, and gives detailed procedures for analysis by both extraction and hematofluorometry.

The concentration of blood lead considered acceptable has been reduced below the point at which EP is affected, which resulted in a decline in demand for this test. EP remains useful, however, in determining the duration and extent of lead exposure, as well as having utility in the characterization of iron status. This NCCLS document provides a thorough discussion of all aspects involved in the laboratory determination of this clinically important analyte.

Key Words

Erythrocyte protoporphyrin, hematofluorometer, iron deficiency, lead poisoning, zinc protoporphyrin.

Erythrocyte Protoporphyrin Testing; Approved Guideline

1 Introduction

Because there is an overall lack of uniformity in measurement practices for erythrocyte proto-porphyrin (EP) testing, the analysis of EP is an area that is in need of guidelines. Differences in the basis for measurement between the two most commonly used methods, extraction and hematofluorometry, have also been a source of confusion. This document provides recommended methods for achieving valid results using either method. To improve comparability between methods, enhance diagnostic utility, and conform to Système International d' Unités (SI units), the subcommittee recommends that all EP measurements be reported in units that compare the abundance of EP to the abundance of heme in the specimen (micromole per mole).

As of 1993, most laboratories were standardizing EP measurements based on an inaccurate reference value. The committee recommends that the dimethyl ester (DME) hydrolysis preparation technique be used for the preparation of protoporphyrin IX (PPIX) standards and that 297 $L \cdot mmol^{-1} \cdot cm^{-1}$ be adopted as the correct millimolar absorptivity ($m\epsilon$) for PPIX. Detailed directions are provided. The revised value for $m\epsilon$ will result in new reference intervals, which are given.

2 Scope

This document is useful to all laboratories that perform EP tests; users of hematofluorometry in nonlaboratory settings will benefit from its use as well.

Recommended methods for extraction (Section 8) and hematofluorometry (Section 9) of EP test procedures are given. The millimolar absorptivity ($m\epsilon$) of protoporphyrin IX (PPIX) is redefined (Section 7). This will result in changes in calibration and reference intervals for EP. New reference intervals are given (Section 10). The history of method development for EP (Section 5) and the biochemistry of porphyrins (Section 4) are described. Terminology (Section 4), sections on the causes of elevated porphyrin results (Section 6) and interpretation (Section 10), and a brief description of liquid chromatography for the differentiation of the various porphyrins (Section 8.9) are provided. The appendices address the preparation of QA materials and the availability of proficiency-testing programs.

Although NCCLS documents generally use units that are fully acceptable within the Système International d'Unités (SI), these do not always coincide with the units recommended by the International Union of Pure and Applied Chemistry (IUPAC) and by the International Federation of Clinical Chemistry (IFCC) for reporting results of clinical laboratory measurements. NCCLS documents also include the IUPAC/ IFCC-recommended units of volume (L) and substance (molecular) concentration (mol/L) in parentheses, where appropriate.

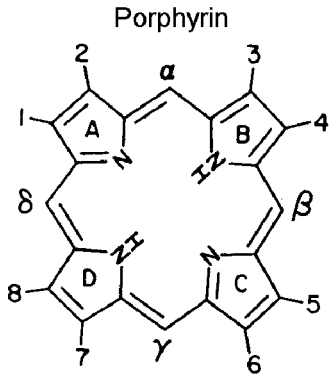
3 Precautions

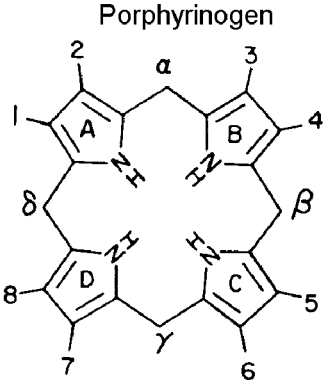
3.1 Universal Precautions

Because it is often impossible to know which might be infectious, all patient blood specimens are to be treated with universal precautions. Guidelines for specimen handling are available from the U. S. Centers for Disease Control and Prevention [*MMWR* 987;36(suppl 2S):2S–18S]. NCCLS document M29-T2, *Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, and Tissue–Second Edition; Tentative Guideline*, deals specifically with this issue.

3.2 Instrument Hazards

As described in the manufacturer's instrument manual, and in relevant sections of this document, when using instrumentation, take precautions to avoid safety hazards and biohazards. For additional information, refer to NCCLS document I17-P, *Protection of Laboratory Workers from Instrument Biohazards; Approved Guideline*.





SUBSTITUENTS IDENTIFYING DIFFERENT PORPHYRINS

Ring	Position	URO	COPRO	PROTO
			Porphyrin III or Porphyrinogen III	
A	1	Acetate	Methyl	Methyl
	2	Propionate	Propionate	Vinyl
B	3	Acetate	Methyl	Methyl
	4	Propionate	Propionate	Vinyl
C	5	Acetate	Methyl	Methyl
	6	Propionate	Propionate	Propionate
D	7	Propionate	Propionate	Propionate
	8	Acetate	Methyl	Methyl
			Porphyrin I or Porphyrinogen I	
D	7	Acetate	Methyl	Does not occur in nature
	8	Propionate	Propionate	

Figure 1. Structure of porphyrin and porphyrinogen and a list of the substituents that identify the different porphyrins. From Labbe RF, Lamon JM. Chemistry of porphyrins. In: Tietz NW, ed. *Textbook of Clinical Chemistry*. Philadelphia: WB Saunders, 1986. Reprinted with permission.

4. Biochemistry

While reading the following sections, refer to Figure 1 for relevant molecular structures and to Figure 2 for an outline of the heme biosynthetic pathway.[1]

4.1 Definitions

Erythrocyte protoporphyrin, *n*- The total non-heme (iron-free) protoporphyrin (3, 7, 12, 17- tetra-methyl-8, 13-divinyl-2, 18-porphine-dipropionic acid) present in erythrocytes. It includes both metal-free protoporphyrin and zinc protoporphyrin (ZP). Erythrocyte protoporphyrin is a product of blood porphyrin analysis in which the commonly used acid extraction solvent liberates the chelated zinc to yield the larger pool of metal-free protoporphyrin. This term is recommended as a replacement for "free erythrocyte protoporphyrin (FEP)," which should be reserved for specific reference to native, metal-free porphyrin.

Ferrochelatase, *n*- The enzyme that catalyzes the chelation of iron (II) by protoporphyrin with the liberation of two protons. Alternatively, the enzyme utilizes zinc ion as the metal substrate in states of insufficient iron. Ferrochelatase can be inhibited transiently in severe, acute lead poisoning. It is not inhibited significantly in chronic lead poisoning **(EC 4.99.1.1)**.

Hematofluorometry, *n*- An analytical procedure based on the measurement of front surface fluorescence of blood (erythrocytes) with exposure to long wavelength ultraviolet

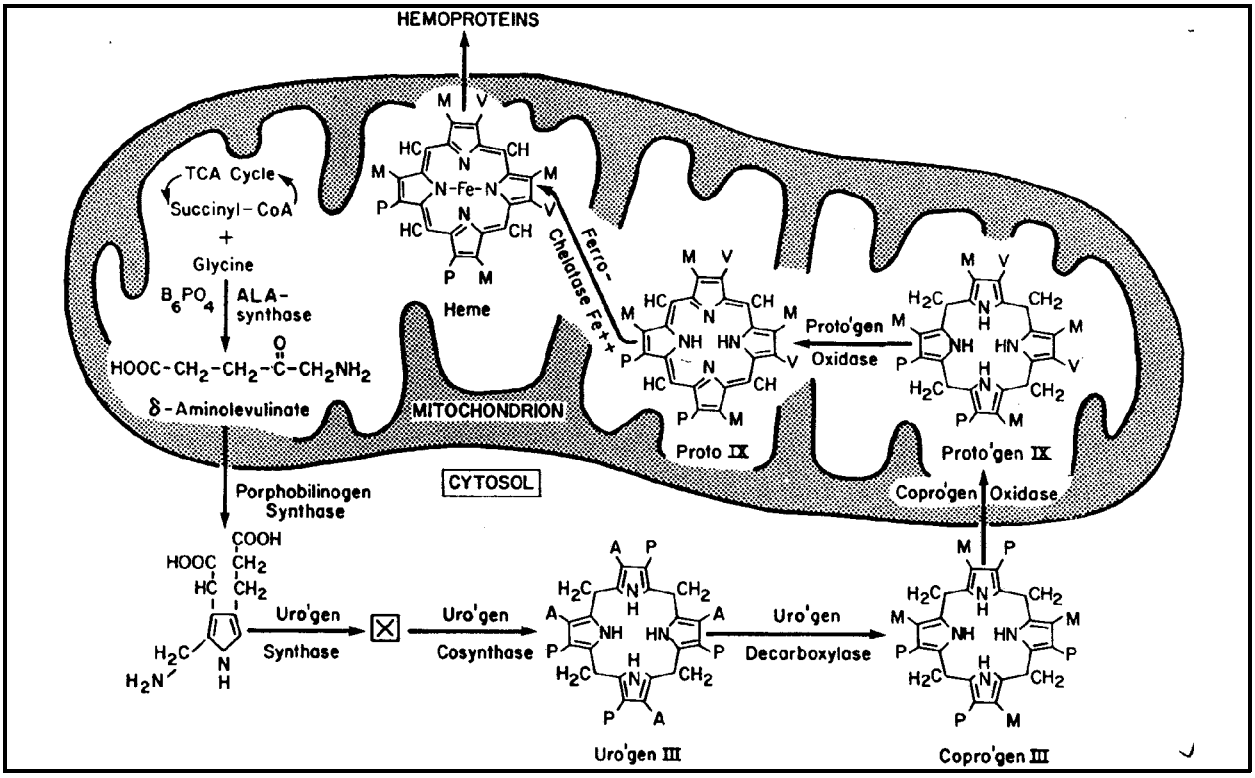


Figure 2. The heme biosynthetic pathway showing the distribution of enzymes and intermediates between the mitochondria and cytoplasm. B_6PO_4 = pyridoxal phosphate. The enzyme uro'gen synthase has recently been termed "porphobilogen synthase." The intermediate, designated by "X" between porphobilinogen and uro'gen III, has been identified as hydroxy methyl bilane. From Labbe RF, Lamon JM. Chemistry of porphyrins. In: Tietz NW, ed. *Textbook of Clinical Chemistry*. Philadelphia: WB Saunders, 1986. Reprinted with permission.

light. The hematofluorometer is a dedicated instrument that is designed specifically to measure the zinc protoporphyrin content of red blood cells (RBCs).

Heme, *n-* Most often refers to iron-protoporphyrin chelates, as in hemoglobin (Hb) or cytochromes. Used generically, heme also designates any iron II (ferrous) porphyrin chelate, e.g., coproheme. Iron III (ferric) protoporphyrin as the chloride is hemin, and as the hydroxide is hematin.

Millimolar absorptivity, *n-* The absorbance of a substance at a concentration of 1 mmol/L, measured with a light path of 1 cm. This absorptivity is a constant characteristic of the absorbing substance and the particular wavelength of radiation used.

Non-heme protoporphyrin, *n-* Refers to all forms of the porphyrins not chelated with either iron (II) or iron (III).

Porphyrins, *n-* Porphyrins are derivatives of porphin, which is a macrocyclic tetrapyrrole bonded by four methane groups. The porphin derivatives have various substituents that occupy the eight peripheral positions on the four pyrrole rings. Of most clinical interest are uroporphyrin (8 carboxyl groups), coproporphyrin (4 carboxyl and 4 methyl groups), and protoporphyrin (2 carboxyl, 2 vinyl, and 4 methyl groups).

Porphyrin precursors, *n-* Include δ-aminolevulinic acid, a product of succinate and glycine condensation/decarboxylation, and porphobilinogen, a monopyrrole formed by the condensation/cyclization of two molecules of δ-aminolevulinic acid.

Porphyrinogens, *n-* Porphyrinogens are reduced, colorless, nonfluorescing forms of porphyrins. They occur as intermediates in heme bio-synthesis. Partially reduced forms are non-fluorescent but are colored.

Protoporphyrin IX (PPIX), *n*- Protoporphyrin IX is derived from series III precursors and it is the naturally occurring isomer (protoporphyrin has 15 possible isomers). It is the immediate precursor to heme. This is commonly but erroneously referred to as the IX isomer.

NOTE: PPIX is the term commonly used in the porphyrin literature and it is the form used in this document. It does not conform to the IUPAC-recommended nomenclature, protoporphyrin 9 (PP 9).

Zinc protoporphyrin, *n*- The naturally occurring byproduct of heme formation that accumulates in increased concentrations when inadequate iron (II) substrate is available for ferrochelatase.

4.2 Abbreviations

DME:	dimethyl ester
EDTA:	ethylenediaminetetraacetic acid
EP:	erythrocyte protoporphyrin
FA:	free acid
Hb:	hemoglobin
HCl:	hydrochloric acid
Hct:	hematocrit
HF:	hematofluorometer
mε:	millimolar absorptivity
PPIX:	protoporphyrin IX
QA:	quality assurance
QC:	quality control
RBC:	red blood cell
λ-max:	wavelength of absorbance maximum
ZP:	zinc protoporphyrin.

4.3 Biosynthesis

Mention of naturally occurring porphyrins first appears in the medical literature late in the 19th century. The Nobel prize was awarded to Hans Fisher in 1930 for the complete organic synthesis of protoporphyrin and heme. Investigations of protoporphyrin biosynthesis began with the advent of radioisotopes in biochemical research in the early 1950s. During the next two decades, the biosynthetic pathway and its regulation were described in detail, largely through the investigations of Neuberger and Shemin and their colleagues. Porphyrin metabolism is now reviewed thoroughly in many excellent references. [2-5]

Intermediates of clinical interest in the porphyrin biosynthetic pathway include the precursors, δ-aminolevulinic acid and porphobilinogen, and the tetrapyrroles, uroporphyrinogen, coproporphyrinogen, and protoporphyrinogen, which lead to the formation of protoporphyrin. In certain acquired and congenital disorders, excess formation and oxidation of the porphyrinogens to porphyrins leads to their deposition in tissues with a resultant photosensitivity. As the terminal reaction of the pathway, the protoporphyrin chelation of iron (II) to form heme is catalyzed by ferrochelatase. When protoporphyrin is being synthesized in the young red cells at a faster rate than iron (II) is being delivered, which is defined clinically as a state of relative iron-deficient erythropoiesis,[4] ferrochelatase can catalyze a greater formation of zinc protopor-phyrin (ZP). Note that lead interferes with iron availability, which might be due to inhibition of iron reductase enzymes.

4.4 Regulation

Primary regulation of the porphyrin/heme pathway occurs at the level of d-aminolevulinate synthetase (EC 2.3.1.37), which is subject to two different influences. The free heme pool in the cell acts either at the transcription level to regulate enzyme formation or through feedback inhibition of preformed enzyme. Although it has been investigated less frequently, evidently a second site of control occurs at the level of porphobilinogen synthase (EC 4.2.1.24; δ-aminolevulinate dehydratase), which is a branch point in metabolism and is influenced by iron status. In pathologic states, genetically altered enzymes can occur and create rather unexpected patterns of porphyrin accumulation and/or excretion. Various feedback inhibitions of pathway enzymes cause these changes, which have much diagnostic importance.

5 History of Analytical Determination of Protoporphyrin

Protoporphyrin assay techniques have developed in response to both perceived needs and instrumentation availability. Initially, studies of blood, urine, and stools from uncommon inborn errors in porphyrin metabolism

(porphyrias) were based on visual absorption or fluorescence emission spectroscopy for semiquantitative assays.[6] Whole blood or washed RBCs were extracted into a 3:1 mixture of ethyl acetate: glacial acetic acid (or ethyl ether: acetic acid) and then into a 5 to 25% hydrochloric acid (HCl) solution to separate fluorescing porphyrins from heme. Colorimetry[7] or spectrophotometry[8] was later employed for quantitative analysis. Schwartz[9] modified the extraction procedure for selective determination of copro- and protoporphyrin. Generally, 2- to 10-mL blood samples were recommended for these procedures.

Increased concentration of red cell porphyrin has long been known to occur in subjects with lead poisoning, and more recent studies show that lead interferes with several steps in heme biosynthesis. An increased awareness of the widespread health hazard associated with increased lead exposure made it necessary to augment blood lead analysis with a rapid, sensitive, and inexpensive screening method to monitor the significant biological effects of lead toxicity. A blood lead determination is an indicator of the dose to which one has been exposed. However, biological variation allows for different tolerances among different persons and segments of the population. Increased EP level has been considered a useful indicator of the effect of lead on the subject at previously accepted, allowable thresholds adopted for lead screening programs. Also, EP proved to be a rapid test that could be conducted in the field with little chance of contamination when compared to blood lead determination.

EP methods quickly evolved. Sassa's adaptation of earlier methods took advantage of the sensitive spectrofluorometers that became available and permitted quantitative analysis of protoporphyrin in as little as 2 μL of blood.[10] The limitation of this method was that it required the use of a research-grade spectro-fluorometer that could not readily be adapted to field use. Piomelli[11] described a micro method, including a dried blood spot filter paper modification, that could be used on a filter fluorometer, which was better adapted for mass screening. Chisolm and Brown[12] further optimized the instrumental parameters and chemical extraction procedures in a "Selected Method."[7] Validation of each step of the extraction and calibration procedures were emphasized.

Procedures for internal QC of both samples and standards were also recommended. In 1976, an Ad Hoc Committee for Methodology and Quality Control for Protoporphyrin IX Standards was convened at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA. The committee was created to establish uniform guidelines for the standardization of extraction procedures and the reporting of test results. It was at this meeting that a $m\epsilon$ of 241 $L \cdot cm^{-1} \cdot mmol^{-1}$ reported by Mauzerall [13] was adopted by consensus.

Another factor that supported the need for a rapid screening technique for EP was the observation that EP concentrations are elevated in cases of iron deficiency.[2] Because this is a common, treatable disorder, much attention has been given to using EP as an indicator of iron deficiency.

An important finding by Lamola et al was that in normal erythrocytes, the major molecular form of protoporphyrin is zinc protoporphyrin (ZP).[14] Previous analytical methods for EP had converted ZP to metal-free EP during the extraction process.

Blumberg et al[15] describe a dedicated front-surface fluorometer that could be used to detect ZP, the major form of protoporphyrin in erythrocytes. This instrument, the hematofluorometer (HF), greatly simplified the analytical procedure and made mass screening in outpatient settings possible. However, proper standardization has been an area of long-standing difficulty[16] (see Section 9).

Chisolm and Brown[17] developed an acetone-extraction procedure specific for ZP. Also, they observed that in many disease states—such as severe lead poisoning, iron deficiency, sickle cell disease, or erythropoietic protoporphyria—a highly variable fraction (approximately 20 to 99%) of the protoporphyrin was not chelated to zinc. They confirmed that this free fraction was contributed by the protoporphyrin found in an increased reticulocyte layer, as reported by Watson et al[18] and Chisolm and Brown[17] report equivalent quantitative results for EP measured by the HCl-extraction method and ZP measured by the acetone-extraction method in reticulocyte-free blood samples.

Poor interlaboratory agreement for EP prompted a reassessment of the published *mε* for PPIX and ZP. If indeed the protoporphyrin being measured by the extraction technique were the same as that measured by the HF, then the values obtained from extraction techniques should have agreed with the ZP values measured by the front surface techniques. However, this was not the case. In 1985, the United States Public Health Service, Division of Maternal and Child Health of the Bureau of Health Care Delivery and Assistance and the CDC consulted with a number of experts in Washington, DC, to review the calibration procedures and the reporting of test results. One recommendation was to re-examine the *mε* for PPIX. In 1989, Gunter et al[19] proposed that the *mε* for PPIX be 297 $L \cdot cm^{-1} \cdot mmol^{-1}$ (see Section 7).

Other investigators described the quantitative determination of metal-free versus ZP.[20] Different species of individual porphyrins can be separated by high performance liquid chromatography (HPLC).[21] These procedures are restricted to research settings and reference laboratories, and they are not available widely for clinical purposes. Therefore, HPLC methods are beyond the scope of this document.

6 Applications of the ErythrocyteProtoporphyrin Test

The concentration of EP is elevated in several pathological conditions that impair the synthesis of heme (Table 1). To make a specific diagnosis, confirmatory laboratory tests are required. The most common use of the EP test is in screening for lead poisoning. Also, EP is increasingly used in screening for iron deficiency because, in contrast to other tests, the analysis can be performed cheaply and rapidly enough by hematofluorometry to determine the need for additional testing during a single patient visit. In addition to lead poisoning and iron deficiency, EP is also elevated in mild and severe inflammatory conditions of more than a few days' duration. Inflammatory disorders that can cause elevated EP include not only chronic illnesses, such as rheumatoid arthritis, osteo-myelitis, and cancer, but relatively acute ones, such as otitis media. One basis for this elevation is the marked decline in serum iron that is associated with inflammatory conditions, which restricts the production of heme and results in the accumulation of EP in RBCs.

Generally, EP is elevated in hemolytic diseases and in chronic blood loss due to conditions, such as peptic ulcers and colon cancer, all of which are typically characterized by an increased rate of RBC production. With increased red cell production, available

Table 1. Conditions Resulting in an Elevated EP Concentration*

Disturbance in the heme synthetic pathway
Congenital
Congenital erythropoietic protoporphyria
Variegate porphyria
Congenital sideroblastic anemia
Acquired
Lead poisoning
Sideroblastic anemia
Increased erythropoiesis (reticulocytosis and/or a relative inadequacy of iron for heme synthesis)
Congenital hemolytic anemias
Acquired hemolytic anemias
Normal newborns
Chronic blood loss
Inadequate iron supply for heme synthesis
Iron deficiency
Inflammatory conditions: infection, collagen disease, Malignancy

*The most common causes are in bold.

storage iron that would meet normal physiologic needs can be inadequate to meet the demands for an ele-vated rate of heme production. Furthermore, the proportion of young red cells becomes increased, and these contain a higher concen-tration of EP than more mature cells. Thus, in iron deficiency, inflammatory conditions, hemolytic diseases, and chronic blood loss, an inadequate iron supply for heme synthesis and/or an increased proportion of young RBCs is responsible for the elevation of EP. On the other hand, in lead poisoning, the interference with later steps in the hemesynthetic pathway results in an elevation of EP.

6.1 Lead Poisoning

6.1.1 Childhood Screening for Lead Poisoning

From 1972 to 1991, EP was the primary screening test recommended by the CDC for childhood lead poisoning detection in the United States.[22-25] EP was most often measured by HF in public health clinics (see Section 9). Because the hematofluorometry test result is available in minutes, a child found to have an elevated EP concentration could have blood drawn at the same visit for a confirmatory blood lead determination. Then, the blood lead and EP concentrations were used to determine the risk level of lead poisoning under the 1978 and 1985 CDC guidelines. In 1978, the CDC defined lead poisoning in children as a blood lead of ≥ 30 μg/dL (1.45 μmol/L) whole blood after a screening EP of ≥ 50 μg/dL (2.40 μmol/L) whole blood.[23] In 1985, the guidelines were revised to define lead poisoning more stringently with a cut-off value for blood lead of ≥ 25 μg/dL (1.21 μmol/L) after a screening EP of ≥ 35 μg/dL (1.69 μmol/L) whole blood.[24] In 1991, a further revision was instituted by the CDC to recommend universal blood lead testing for infants and young children and to define lead poisoning as a concentration of blood lead of >10 μg/dL (0.48 μmol/L).[25] At this blood lead concentration, the diagnostic sensitivity and specificity of the EP test for lead poisoning is poor.[26]

Consequently, the CDC no longer recommends EP as the primary screening test to identify children with lead poisoning; instead, the routine determination of blood lead in young children is advocated.[25] It remains to be seen to what extent universal blood lead testing, as recommended by the CDC, will be put into practice. If the recommendations are fully implemented, the future role for EP in the management of lead poisoning is likely to be as follows:

- Screening
  - As a secondary test to blood lead for prioritizing management when blood lead concentration is between 10 and 20 μg/dL (0.48 and 0.97 μmol/L).
  - To identify concomitant iron deficiency.
- Diagnosis
  - In addition to blood lead content, to evaluate the biological effects of lead poisoning.
  - To monitor cases under medical management.

6.1.2 Occupational Lead Poisoning

Protoporphyrin measurement is widely used, in conjunction with the blood lead concentration, for monitoring the lead status of industrial workers who are exposed to lead. Measurement of EP (specifically ZP) level,** together with blood lead concentration, is part of the Occupational Safety and Health Administration (OSHA)-required initial baseline for lead-exposed workers, who should be subsequently tested every 6 months.[27] However, the specific guideline for action is based not on the EP, but on the blood lead concentration alone. A worker who is found to have a high blood lead concentration will be removed from the area of lead exposure and required to have serial blood lead and EP measurements to monitor improvement and to determine a suitable time for returning to the work setting. Currently, the blood lead threshold concentration for removing a worker from an area of lead exposure is ≥ 60 μg/dL (2.90 μmol/L) on a single occasion, or three sequential measurements that average ≥ 50 μg/dL (2.40 μmol/L). The criterion for per-mission to return to the lead exposure area is a blood lead concentration of < 40 μg/dL (1.93 μmol/L).[27] At these blood lead

**Occupational Health and Safety Administration regulations specify ZP as the entity to be measured, but no threshold values are specified.

concentrations, EP has a strong correlation with blood lead and can serve as part of the monitoring system for recovery from occupational lead exposure. An action concentration for EP in occupational lead exposure still needs to be defined. EP is a useful physiological marker for the biologically harmful effects of lead because elevated EP levels signify interference with the heme-synthetic pathway.

6.2 Iron Deficiency

6.2.1 Screening for Iron Deficiency

Measurement of EP level is one of several tests that can be used to detect iron deficiency anemia or a milder form of iron deficiency without anemia that is characterized, nevertheless, by impaired heme synthesis. The EP test is used mainly in screening children.[28-30] A therapeutic trial of iron may be considered when the EP level is elevated in combination with an abnormally low Hb, hematocrit (Hct), or serum ferritin level. A significant reduction of EP concentration and/or an increase in hemoglobin concentration after a month of iron treatment suggests the earlier presence of iron deficiency. In adults, the EP test is used primarily to screen for iron deficiency in blood donors.[31,32] Similarly, it is used to screen for iron deficiency[33] in patients with renal disease who are undergoing repeated hemodialysis.[34] EP is useful for screening pregnant women for iron deficiency because EP values remain stable during pregnancy if there is adequate iron nutrition,[35] whereas other measures of iron status change.[36]

6.2.2 Diagnosis of Iron Deficiency

EP is one of three biochemical tests, together with serum ferritin and transferrin saturation (serum iron/iron binding capacity), that can be used in the diagnosis of iron deficiency.[37,38] Any of these tests can provide additional evidence for the presence of iron deficiency after a patient is found to be anemic on screening. However, no single one of these tests, in the absence of anemia, has a high sensitivity or specificity for iron deficiency.[39] All three tests may be used in combination with hemoglobin in nutrition surveys[38,39] and for research involving iron nutrition [28,29,37] where the extra expense can be justified for the purpose of providing greater certainty about iron status.

6.2.3 Differential Diagnosis of Microcytic Anemia

The determination of EP is helpful in differentiating the two major causes of microcytic anemia (anemia with a low mean corpuscular volume): iron deficiency anemia and thalassemia trait.[40] Typically, iron deficiency is associated with an elevated EP level, whereas, usually, the EP level is normal in anemia of both alpha- and beta-thalassemia traits.

6.2.4 Inflammatory Conditions

A variety of inflammatory conditions can complicate the interpretation of an elevated EP level in persons who are being screened for lead poisoning and/or iron deficiency. This is most evident among infants and young children who often have, or have recently had, mild infections (e.g., upper respiratory infections or otitis media) of more than a few days' duration[41]; also, it can be a problem among elderly persons, many of whom have severe or mild chronic inflammatory conditions, such as arthritis.[42]

6.2.5 Porphyrinopathies

Porphyrias are inherited or acquired disorders of porphyrin metabolism that result in the elevation of one or more of porphyrin compounds.[43] Porphyrinopathies include both the porphyrias or inherited (primary) disorders and the induced (secondary) disorders in porphyrin metabolism. In some porphyrias, the formation, accumulation, and excretion of porphyrins can be extremely elevated.

6.2.5.1 Hereditary Porphyrias

At least seven varieties of hereditary porphyrias have been identified, each being associated with a different enzyme defect in the hemesynthetic pathway, with the exception of δ-aminolevulinate synthetase.

Also, the porphyrias differ with respect to the tissue in which the enzyme becomes rate-limiting, specifically, bone marrow (erythropoietic origin) or liver (hepatic origin). Even though all porphyrias are characterized by an elevation of some type of porphyrin, only certain porphyrias are associated with a significant elevation of EP level: erythropoietic protoporphyria (hepatic and erythropoietic origin), variegate porphyria (hepatic origin), porphyria cutanea tarda, and copro-porphyria.[43,44]

6.2.5.2 Acquired Porphyrias

Some porphyrinopathies are related to non-congenital disease or to chemical exposure.[45] However, with the exception of lead, most of the these are characterized by an elevation of porphyrins other than protoporphyrin IX. Lead poisoning is considered to be a form of acquired porphyria.

6.2.6 Sideroblastic Anemias

Sideroblastic anemias are a heterogeneous group of disorders of heme synthesis characterized by ineffective erthyropoiesis. As in the porphyrias, there are congenital and acquired forms. The condition is characterized by anemia, the presence of ring sideroblasts, and accumulation of metal-free EP. [44]

7 Millimolar Absorptivity: A Historical Perspective

It is conventional analytical practice to establish standard concentrations by using a known mass of analyte. This is not possible with PPIX because of impurities and stability limitations. Therefore, an approximate mass is assumed, which must be corrected using molecular absorption spectrophotometry and Beer's law. This requires accurate knowledge of the *m*ϵ of the substance being analyzed.

Previous determinations of the *m*ϵ for PPIX might have been compromised by the use of impure source materials. In addition, use of slit widths >10 nm for establishing absorbance wavelength maximum (λ-max), variability in HCl concentration of the solvent, and the fact that solutions of PPIX with identical theoretical concentrations can exhibit different absorbance values depending on their initial preparation, have made it difficult to determine the correct value. In the past, interlaboratory agreement for the determination of EP levels has been somewhat problematic because of the wide-spread use of secondary standards, such as coproporphyrin, discrepancies in the *m*ϵ value for PPIX, and the issue of extraction efficiency.

Following the measurement of a 1-mg/L solution of PPIX in HCl, the following *m*ϵ were reported:

*m*ϵ	HCl Concentration	λ-max	Reference
278	8.3 mol/L	411.0	Grinstein, 1948[46]
262	2.7 mol/L	408.0	Rimington, 1960[47]
275	1.5 mol/L	408.0	Schwartz, 1960[9]
241	1.0 mol/L	408.0	Nat Acad Sci, 1972[13]
297	1.5 mol/L	408.0	Gunter, 1989[19]

However, the most frequently used EP micromethods[10,11] measured PPIX standard concentration in 1.5 mol/L HCl, not 1.0 mol/L. Also, use of a convenient "5-μg" PPIX tube standard as a primary standard source contributed error, because the amount of PPIX contained in the tube was not truly 5 μg; rather, it delivered an amount calculated to produce a solution with an absorbance corresponding to an expected concentration of 5 μg per 10 mL, if a *m*ϵ of 241 $L \cdot mmol^{-1} \cdot cm^{-1}$ is assumed.[19]

PPIX tends to form molecular aggregates in dilute aqueous solutions[48]; this aggregation is associated with a decrease in the *m*ϵ and region broadening in the Soret band.[49] One-mg/L solutions of PPIX in 1 mol/L HCl prepared directly from PPIX free acid (FA) exhibit an *m*ϵ equal to 272 $L \cdot mmol^{-1} \cdot cm^{-1}$.[19] When prepared from a hydrolysate of PPIX dimethyl ester (DME), the *m*ϵ is equal to 297 $L \cdot mmol^{-1} \cdot cm^{-1}$.[19] This difference can be explained by aggregation because the approximately 10% difference in absorbance of these two solutions is matched by a 10% difference in fluorescence. The greater the degree of polymerization, the lower the level of fluorescence or absorbance is observed in solution. Apparently, hydrolysis results in minimal aggregation, which creates a more dispersed state of PPIX molecules by providing the most monomeric form of PPIX.[19]

To establish a clinical database of EP concentrations for use in childhood lead poisoning screening programs, adoption of a *m*ϵ of 241 L · mmol^{-1} · cm^{-1} was recommended as an arbitrary uniform standard value in 1975.[12] This recommendation was echoed in a meeting of protoporphyrin researchers at the CDC in 1976. However, data from the United States National Normative and Prevalence Studies, the Second National Health and Nutrition Examination Survey (NHANES II, 1976–1980),[50,51] the Hispanic Health and Nutrition Examination Survey (HHANES, 1982–84),[52] and NHANES III (1988– 1994), determined with PPIX standards prepared from hydrolysates of DME, demonstrated approximately 19% lower EP concentrations than previously published studies that used the 241 *m*ϵ value.

The discrepancies in the results of these studies have led to the proposal, in 1989, for the formal establishment of the *m*ϵ (of a 1-mg/L solution of PPIX in 1.5 mol/L HCl at 408.0 nm with a 0.5-nm bandpass) as 297 L · mmol^{-1} · cm^{-1}. The effect on the public health of revising the *m*ϵ would be a 19% decrease in the current clinical reference intervals for EP values [e.g., this would have changed the 1985 CDC-recommended EP cut-off value[24] from 35 to 28 μg/dL (0.62 to 0.50 μmol/L) of whole blood in childhood lead poisoning programs]. Data generated by other laboratories using an *m*ϵ of 241 L · mmol^{-1} · cm^{-1} can be converted to the recommended *m*ϵ of 297 by multiplying by 241/297 in order to compare it to the NHANES national databases and future databases using the correct mϵ.

After reviewing the data, the subcommittee recommends the adoption of an *m*ϵ of 297 L · mmol^{-1} · cm^{-1}. Furthermore, the subcommittee recommends that the DME-hydrolysis preparation technique for stock standards of PPIX be used as the preferred method of standard preparation (see Section 8.3.)

Adoption of mϵ 297 L·mmol^{-1}·cm^{-1} will significantly change the reference ranges for EP. Revised reference intervals are provided in Section 10.2. Note that this standardization change, and the associated effect on results and reference intervals, must be clearly communicated to the practicing clinician to prevent incorrect interpretation of EP results.

8 Recommended Procedure for the Quantitative Fluorometric Determination of Erythrocyte Protoporphyrin After Extraction

8.1 Principle of the Method

Porphyrins and heme components are extracted from anticoagulated whole blood into an ethyl acetate–acetic acid mixture. Next, protoporphyrin is back-extracted into an HCl solution. In low pH aqueous solutions, ZP dissociates into Zn^{2+} and protoporphyrin IX free acid (PPIX FA); then, the concentration of PPIX in the

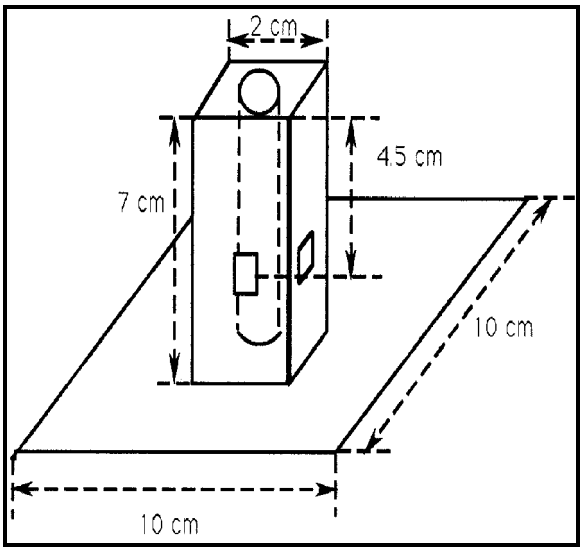


Figure 3. EP cell holder

aqueous phase is measured by molecular fluorometry. Fluorescence is a relative measurement and standards are critical. The calibration curve is based on dilutions of a PPIX standard, the concentration of which is first established by molecular absorption spectroscopy.

This description is based on a method jointly developed by the New York State Department of Health (Parsons PJ, personal communication, 1988), the Wisconsin State Laboratory of Hygiene (Stanton NV, personal communication, 1988), and the CDC.[52]

8.2 Apparatus

8.2.1 Instrumentation

The following instrumentation is necessary for the quantitative determination of erythrocyte protoporphyrin after extraction:

- A spectrofluorometer, with a bandpass of 5.0 nm or less at 640–670 nm, equipped with a red-sensitive photomultiplier tube with S-20 response, and fitted with a cell-holder (see Figure 3) adapter for 10-X 75-mm glass tubes and a suitable printer or digital display
- Centrifuge (general laboratory) -uipped for 10- X 75-mm tubes
- Vortex mixer
- Analytical balance
- Chemical fume hood
- Automated dispensers and dilutors (or fixed-volume bottle repetitive dispensers, and micropipettors (50 μL, 100 μL, 200 μL, 400 μL, and 500 μL)
- Magnetic stirrer and stir bars
- Spectrophotometer, UV-visible, double-beam, with variable slit-widths adjustable to 0.5 nm at 405–410 nm, equipped with 1-cm quartz cells.

8.2.2 Chemicals

The following chemicals are necessary:

- Deionized water, greater than or equal to 1.0 megohm-cm at 25 ° C, treated to remove organic compounds, with zero background fluorescence
- Protoporphyrin IX dimethyl ester (PPIX DME), $C_{36}H_{38}N_4O_4$, ≥ 99% purity
- Ethyl acetate, $CH_3CO_2C_2H_5$, high-purity (see Section 8.8.2)
- Acetic acid, CH_3COOH, glacial, ACS grade
- Hydrochloric acid, HCl, concentrated, ACS grade
- Formic acid, CH_2O_2, concentrated, ACS grade
- Potassium iodide, KI, ACS grade (see Section 8.8.2).

8.2.3 Glassware

The following pieces of glassware are necessary:

- Volumetric flasks, actinic, class A, with stoppers (1 L, 500 mL, 250 mL, 200 mL, 100 mL, 10 mL) (for class A flask volume tolerances see ASTM standard #E288[53]) and clear glass, 1 and 2 L. If actinic glass is not available, clear glassware wrapped in aluminum foil may be substituted.
- Pipets, volumetric, class A (25.0 mL, 10.0 mL, 8.0 mL, 6.0 mL, 5.0 mL, 4.0 mL, and 2.0 mL) (for class A volumetric pipet tolerances see ASTM standard #E969[54])
- Culture tubes, glass, disposable, 10- x 75-mm
- Graduated cylinders, 1 L, 250 mL
- Microtiter plates, plastic, 96- x 0.5-mL wells (optional: 10- x 75-mm glass culture tubes may be substituted).

8.2.4 Reagents

The following reagents are necessary:

8.2.4.1 Hydrochloric Acid Solutions

NOTE: Although concentrated HCl is commonly assumed to be 12 mol/L, each lot can vary slightly upon manufacture. The correct substance (molar) concentration should be determined using the following formula:

$$\text{mol/L} = \frac{\text{Density g/mL} \bullet \%\text{HCl}}{36.435\ \text{g/mol}}$$

Information on the density and percentage of HCl in the assay should be provided on each bottle label.

The following directions are based on a lot of concentrated HCl determined to be 12.7 mol/L. Laboratorians should verify the molarity of their particular lots and adjust required acid volumes accordingly before beginning protoporphyrin analysis.

Laboratorians should always wear eye protection and acid-resistant gloves when working with HCl, and they should always remember to add acid to water when preparing solutions.

- Following are directions on how to prepare HCl, 7.0 mol/L (for hydrolysis):

(1) Add 138 mL of concentrated HCl (12.7 mol/L) to 100 mL of deionized water in a volumetric flask.

(2) Dilute the mixture to 250 mL.

- Perform the following steps to prepare HCl, 1.62 mol/L (for daily absorbance readings at a final concentration of 1.5 mol/L):

(1) Add 141 mL concentrated HCl to 250 mL deionized water in a volumetric flask.

(2) Dilute the mixture to 1.0 L.

- Perform the following steps to prepare HCl, 1.5 mol/L (for analysis/extraction and for blanking spectrophotometer):

(1) Add 118 mL concentrated HCl to 250 mL deionized water in a volumetric flask.

(2) Dilute the mixture to 1.0 L.

8.2.4.2 Ethyl Acetate, 2:1 (v/v)

To prepare ethyl acetate, 2:1 (v/v):

(1) While working under a hood, combine 400 mL of ethyl acetate and 200 mL of glacial acetic acid in a reagent bottle.

(2) Mix the solution well, using a clean glass rod or by capping the bottle and swirling it thoroughly. This volume is sufficient for the complete assay of standards, controls, and 80 specimens in duplicate. Larger volumes may be prepared weekly and stored in amber bottles.

8.3 Protoporphyrin IX Standard Solutions

To minimize photodecomposition of PPIX, all standard solutions are prepared in actinic glass-ware or in clear glassware wrapped in aluminum foil.

The standard preparation of PPIX DME by hydrolysis is described. Perform all work under yellow-filtered lights, if possible; if not, fluorescent lighting should be reduced and sunlight avoided because the standard solution is photo-chemically unstable. Darkroom filters for fluorescent tubes are ideal for this purpose. Because solutions of PPIX increase in both fluorescence and absorbance at colder temperatures, bring all solutions to $20 \pm 2\ ^{\circ}C$.[19]

(1) Protoporphyrin IX Free Acid Hemolysate Stock Solution, 200 mg/L

The hydrolysis step produces PPIX from the stable DME form in the presence of strong acid.

(a) Weigh 42.0 mg PPIX DME.

(b) Transfer the PPIX DME to a 200-mL volumetric flask containing 100 mL of 7.0 mol/L HCl, and wash the PPIX off the weighing paper with a few drops of formic acid for quantitative removal.

(c) Dilute to volume with 7.0 mol/L of HCl solution.

(d) Add a small acid-rinsed stirring bar. Mix the contents at ambient temperature for 3 hours, using a magnetic stirrer.

(e) Proceed to step 2a.

Prepare stock solution as needed. Use once and discard, after the subsequent dilution is prepared. Hydrolysis of PPIX DME for >3 hours leads to the formation of degradation products, such as hematoporphyrin, which causes inaccuracy in absorbance values and subsequent concentration determinations.[55]

Purchase of a large quantity (>5 g) of PPIX DME is recommended. Material may be aliquoted into 50-mg quantities, placed into sealable glass vials or ampules, preferably under inert gas, and stored at −70 °C for maximum stability and ease of use.[52]

(2) Protoporphyrin IX Intermediate Stock, 10 mg/L, in 0.35 mol/L HCl

(a) After 3 hours of mixing, add 25.0 mL of stock solution to 300 mL of deionized water in a volumetric flask.

(b) Dilute to 500 mL.

Prepare as needed. At this lower HCl concentration, the standard is stable for extended periods compared to 1.5 mol/L HCl concentrations.[19] For maximum stability, the solution should be stored at 4 °C and wrapped in aluminum foil. Properly prepared and stored solutions are stable for at least 1 month.

(3) Protoporphyrin IX Intermediate Working Stock Standard, and Daily Absorbance and Concentration Verification Standard, 0.5 mg/L in 1.5 mol/L HCl

(a) Bring intermediate stock solution to ambient temperature (20–25 °C).

(b) Transfer 5.0 mL of 10.0 mg/L PPIX solution to a volumetric flask containing 50 mL 1.62 mol/L HCl.

(c) Dilute to 100.0 mL with 1.62 mol/L HCl.

Prepare daily to verify absorbance of standard solution, blanked against 1.5 mol/L HCl. Scan between 395 and 420 nm and record the peak absorbance A (λ-max), relative to the HCl blank. Record the absorbance of this solution to be used in the calculations (Section 8.6.1). Calculate the corrected concentration of the solution by multiplying the absorbance value by 1.89. Use this value in the specimen-concentration calculations (refer to Section 8.5.1).

The theoretical concentration of this solution with respect to PPIX FA is calculated as follows:

$$\text{(a) } 42\ \text{mg PPIX DME} \cdot \frac{562\ \text{mg PPIX FA}}{591\ \text{mg PPIX DME}} = \frac{0.20\ \text{mg PPIX FA}}{200\ \text{mL}} = \frac{0.20\ \text{mg PPIX FA}}{\text{mL}}$$

$$0.20\ \text{mg PPIX FA} = 200\ \mu\text{g PPIX FA}$$

$$\text{(b) } \frac{200\ \mu\text{g PPIX FA}}{1\ \text{mL}} \cdot \frac{25\ \text{mL}}{500\ \text{mL}} \cdot \frac{5\ \text{mL}}{100\ \text{mL}} = \frac{0.50\ \mu\text{g PPIX FA}}{\text{mL}}$$

$$\text{(c) } \frac{0.50\ \mu\text{g}}{1\ \text{mL}} \cdot \frac{1\ \text{mmol}}{562{,}000\ \mu\text{g}} \cdot \frac{1000\ \text{mL}}{1\ \text{L}} = \frac{8.9 \cdot 10^{-4}\ \text{mmol}}{\text{L PPIX FA}}$$

When checking a fresh hydrolysate, verify the purity of the standard with the spectrofluorometer by setting excitation at 408.0 nm and scanning for emission peaks at approximately 655 to 665 nm. Although an emission peak at approximately 607 nm is more intense than the broader peak at 662 nm, it is also more subject to interferences.[9]

(4) Working Standards

(a) Using fixed-volume or adjustable micropipets, or automated pipettors,

transfer 8.0, 6.0, 4.0, 2.0, and 1.0 mL of the 0.5-mg/L PPIX solution into five 10-mL volumetric flasks labeled S_5, S_4, S_3, S_2, and S_1. (The sixth flask serves as a blank, S_0.)

(b) Dilute to 10.0 mL with 1.5 mol/L HCl.

(c) The resulting concentrations are 400, 300, 200, 100, 50, and 0 mg/L PPIX. After dilution during analysis, the final concentrations are equivalent to 2000, 1500, 1000, 500, 250, and 0 μg/L of whole blood.

(d) Transfer 50 μL of each standard to triplicate 10- x 75-mm culture tubes.

This standard dilution protocol has also been modified to be fully automated.[52]

8.4 Specimen Requirements

Specimens should be well-mixed, anti-coagulated whole blood. The Na and K salts of ethylenediaminetetraacetic acid (EDTA) are considered a superior anticoagulant to heparin or citrate because it prevents the formation of microclots upon storage, and because the specimen collected may be analyzed for EP, blood lead, and complete blood counts. Specimens should be collected according to the following options. If the EDTA is provided in liquid form, dilution effects are possible. Refer to NCCLS guideline H35-P, *Additives to Blood Collection Devices: (EDTA); Proposed Guideline*, for more information.

- For fingerstick collection, use plastic microcontainers, with EDTA or heparin anticoagulant. If samples are not to be analyzed or shipped immediately, they should be refrigerated and analyzed within 1 week, unless the laboratory has established other stability criteria.

- For venous specimens, use an EDTA-anticoagulated evacuated specimen collection tube, and refrigerate until it is assayed locally within 2 weeks. If analysis cannot be performed within that time interval, transfer a 0.5-mL aliquot to a high-density, screw-capped, polypropylene tube, and freeze at −20 °C until analysis. (For safety reasons, glass evacuated tubes should not be used for frozen storage. Glass tubes are prone to breakage if frozen improperly.)

Gloves must be worn and appropriate precautions taken when handling blood specimens. Chemicals should be confined to an exhaust ("fume") hood, as much as possible. All materials must be disposed of properly, bearing in mind both biohazard and environmental safety concerns.

8.5 Method for the Determination of Erythrocyte Protoporphyrin by Extraction

For the purposes of this document, this procedure is based on a consensus method developed by the New York State Department of Health (Parsons PJ, personal communication, 1988), the Wisconsin State Laboratory of Hygiene (Stanton NV, personal communication, 1988), and the CDC.[52]

8.5.1 Sample Preparation for Specimens and QC Materials

(1) Thaw frozen specimens and QC materials before analysis. Mix blood specimens thoroughly before sampling. Blood in microcontainers should be vortexed to ensure complete mixing. Venous specimens should be rotated or rocked for several minutes.

(2) Pipet 200 μL of deionized water into a 10- x 75-mm culture tube or microtiter plate well.

(3) Withdraw 50 μL blood from the specimen using a micropipettor or an auto-dilutor. Fibrin clots should be avoided during sampling.

(4) Deposit the blood into the tube or well and mix thoroughly by withdrawing and dispensing the blood–water mixture several times until the solution in the tip is homogeneous and empty. If a culture tube has been used, vortex again at this time. (This dilution

procedure will ensure complete hemolysis of fresh whole blood.)

(5) Transfer 50 µL of the diluted blood into a separate 10- x 75-mm culture tube.

An alternative scheme for sample dilution is to use an automated pipettor capable of accurately measuring 10 µL and adding this volume of blood to 40 µL of water in a glass culture tube.

NOTE: Standards are treated identically to the specimens throughout the procedure, although they are not initially diluted 1:5 as are the specimens.

8.5.2 Extraction Procedure for Standards and Samples

Following is the extraction procedure for standards and samples:

(1) Prepare blanks and standards in triplicate. The number of replicates for QC materials and specimens should be the same. The necessary number of replicates should be established in the laboratory. Remember that 10- x 75-mm tubes may display slight variation from tube to tube, because they are not optically perfect cuvettes. Prepare several reagent blanks with no specimen added.

(2) Add 1 mL of ethyl acetate/acetic acid reagent (see Section 8.2.4.2) to each of the culture tubes containing 50 µL of diluted blood, standard, or QC pool.

(3) Vortex the contents of tube gently for 10 seconds.

(4) Add 1.0 mL of 1.5 mol/L HCl solution.

(5) Cap the tubes (or wrap the tube top with laboratory film) and vortex it thoroughly for 10 seconds.

(6) Centrifuge all tubes for 4 minutes at 1800 x *g*.

(7) Wipe the outside of each tube with a laboratory tissue to remove dust, fingerprints, or grease before inserting the tube into the cell holder of the fluorometer.

8.5.3 Fluorometric Readings

Refer to the instrument manual for specific operating instructions.

(1) Allow 30 to 60 minutes for the spectro-fluorometer to stabilize after the xenon lamp has been ignited. Proper lamp alignment should be verified periodically (at least every 6 months). Set slit widths to 5.0 nm.

(2) Set the excitation wavelength to 408 nm, using the highest concentration working standard, and verify the emission maximum by scanning between 655 and 665 nm.

(3) Use one of the reagent blank solutions to set zero. Discard the standards used to set up the instrument.

(4) Check that the reagent blanks have no background fluorescence relative to deionized water, then re-zero the spectro-fluorometer using the reagent blanks.

(5) Record the fluorescence intensities of each tube.

(6) Specimens or control materials with readings higher than that of the highest standard (S_5) concentration require dilution. Transfer 500 µL of the aqueous phase into a second 10- x 75-mm culture tube. Add 2 mL of 1.5 mol/L HCl, vortex, and measure the fluorescence intensity of this diluted sample.

Correct the calculated concentration by multiplying by 5.

NOTE: Specimens with fluorescence intensity readings exceeding those of the highest standard can demonstrate a quenching effect; therefore, the sample must be diluted to fall within the linear range.[56]

(7) Properly dispose of contaminated waste by putting it in biohazard bags and containers. Organic solvent waste may be pooled carefully in original bottles for disposal by a certified contractor.

8.6 Calculations

8.6.1 Correction of Nominal 0.5 mg/L Standard for Actual Concentration

Use the following equation (Beer's Law):

$A = m\epsilon bc$

where:

A = observed absorbance

b = cuvette path length, 1 cm
c = concentration in mmol/L

$m\epsilon$ = 297 L · mmol^{-1} · cm^{-1}, the millimolar absorptivity of PPIX FA in 1.5 mol/L HCl.

By rearrangement:

$$\frac{0.5 \text{ mg PPIX}}{\text{L}} \cdot \frac{1 \text{ mmol}}{562.3 \text{ mg}} = \frac{8.992 \cdot 10^{-4} \text{ mmol}}{\text{L}}$$

$$\text{Nominal Abs.} = \frac{297 \text{ L}}{\text{mmol-cm}} \cdot 1 \text{ cm} \cdot \frac{8.992 \cdot 10^{-4} \text{ mmol}}{\text{L}}$$

$$= 0.2641$$

Therefore, the true concentration equals:

$$\frac{0.5 \text{ mg/L} \cdot \textit{Measured A}}{0.2641}$$

Or equals: 1.89 · A.

8.6.2 Working Standard Concentrations

Following are the working standard concentrations:

Standard Stock Conc · Dilution = Corrected Stock

	Ratio	Conc
S_5	1.89 · A · (8/10)	= 400 µg/L
S_4	1.89 · A · (6/10)	= 300 µg/L
S_3	1.89 · A · (4/10)	= 200 µg/L
S_2	1.89 · A · (2/10)	= 100 µg/L
S_1	1.89 · A · (1/10)	= 50 µg/L.

PPIX Concentration in Specimens:

(1) Perform a linear regression with x equal to the corrected standard concentration and y equal to the fluorescence intensity to compute a least squares line of best fit. Using the equation of this line, calculate the initial concentration of the PPIX/L of whole blood for each specimen.

(2) Multiply the initial concentration by the correction factor for the blood–water dilution ratio:

PPIX (uncorrected) · 5.0 = PPIX µg/L (whole blood).

8.7 Reference Intervals

For interpretation of results and conversion to other units, refer to Section 10. For establishing appropriate reference intervals, refer to NCCLS document C28-A, *How to Define and Determine Reference Intervals in the Clinical Laboratory; Approved Guideline*.

8.8 Quality Assurance

8.8.1 Quality Control (QC) Materials

QC materials must be included with each batch of EP determinations. Use of at least two different levels spanning the normal to elevated concentration range is recommended. The handling of specimens and controls should be identical, including the number of replicate analyses.

Commercially prepared QC materials are available. However, if resources permit, QC pools may be prepared in house (see Appendix A). Blood from healthy human donors may be used for the normal level pools. For elevated concentration pools, it is preferable to have a blood matrix in which the PPIX is elevated in vivo (rather than by spiking, which can result in poor stability) using animal sources that emulate

lead-exposed or iron-deficient human donors. Often, the preparation of PPIX control materials can be incorporated into the preparation of those needed for blood lead. Appropriate QC materials may be prepared as described in Appendix A.

8.8.2 Additional Analyte Considerations

Following are some additional analyte considerations:

- Coproporphyrin is not appropriate for use as a standard. However, coproporphyrin and Rhodamine B can be used to check instrument performance.

- Interferences

Because the PPIX is separated by extraction from blood components, few interferences exist for this method.

Interferences—such as bilirubin, increased hemoglobin, riboflavin, quinine, and other fluorescing drugs—which have been reported to interfere with the hematofluorometric measurement technique for ZP, do not affect extraction EP measurements. The Stokes shift occurs at about 200 nm for porphyrins, which means there are few biological interferences for protoporphyrin. Heme absorbs in the Soret region (395–425 nm), so excess heme in the sample that is not cleanly extracted could interfere. Heme acts as a filter and absorbs excitation energy, causing a negative interference.

- Potassium Iodide Test

Ethyl acetate quality appears to be the most frequent source of problems in performing the assay. Grade is not as critical as is the fact that the reagent must pass the potassium iodide (KI) test for quenching agents. Test each new bottle of ethyl acetate for the presence of impurities, such as peroxides, which may quench PPIX fluorescence. Working in a chemical fume hood, transfer 50 mL of ethyl acetate into a glass beaker. Place the beaker on a white background and add 10 mL of 10% (w/v) KI in a deionized water solution and gently swirl the contents of the beaker. The presence of a distinct yellow color indicates impurities, which can result in out-of-control QC material values, usually with a negative bias. In the event that pure supplies of ethyl acetate are unavailable, redistillation of the solvent can be necessary. Consult standard texts on practical organic chemistry before attempting redistillation of ethyl acetate, because this is a highly flammable solvent. Smaller bottles (e.g., 500 mL) are optimal; larger-volume bottles might pass the KI test initially, but the quality of the ethyl acetate declines as the bottles are opened repeatedly for use.

- Use of disposable 10- x 75-mm culture tubes, rather than 1-cm square cuvettes, is a practical accommodation to improve the sample throughput and because of the corrosive nature of the extracted samples. Use only high-quality tubes, and verify the suitability of new lots by checking groups of randomly selected tubes for the precision of fluorescence intensities.

- Use of a constant temperature range (refer to Section 8.3) for the standard solutions is critical, because both fluorescence and absorbance of PPIX are inversely proportional to temperature.[46]

- Spectral band-passes greater than 5.0 nm can give falsely elevated fluorescence intensity values.[57]

- Clean any spilled analytical material immediately because the extraction solution is corrosive.

- For older fluorometers that lack an ozone-conversion system, place the xenon lamp under an exhaust hood or snorkel, if possible. Newer instruments have ozone converters to eliminate this problem.
- Monitor lamp and photomultiplier performance by measuring the signal-to-noise ratio of the blank sample.

- Instruments should be maintained according to all ASTM E388-72 guidelines.[57]

- Monitor a chart recorder output for an excessively noisy baseline, or inability to set the blank correctly.

8.9 HPLC Analysis

Differential quantitation of various porphyrins in blood is essential for confirming conditions other than lead poisoning or iron deficiency anemia, which are characterized by an elevation of ZP. Other disturbances of heme biosynthesis occur in congenital porphyrias, chemically induced porphyrias, acute porphyrias, porphyria cutanea tarda, and various anemias. Confirmation of other porphyrias may be made with urine or stool specimens.
Use of HPLC allows the advantage of specification and quantitation of porphyrin forms.

Usually, fluorometric detection is used after separation by isocratic, reversed-phase, ion-pair chromatography. Blood samples are extracted with or without subsequent esterification. Because of its inherently greater complexity, cost, and much slower throughput, this technique is generally reserved for research purposes, or for confirmation and differential diagnosis of suspected porphyrias, rather than for lead-screening programs.[58-60]

9 Front-Surface Fluorometry (Hematofluorometry)

9.1 Principle of Operation

Hematofluorometers (HFs) are portable devices dedicated to one specific analysis. Originally, they were designed for the direct determination of ZP in a single drop of blood.[15] Also, some instruments have been developed for the determination of bilirubin[61,62] and hemoglobin.[63] The hematofluorometer operates on the principle of front-surface (or front-face) fluorometry. Detailed descriptions of the original prototype instrument developed by researchers at the Bell Laboratories can be found in the literature.[15,64] In a conventional spectrofluorometer, the incident and emitted light paths are at right angles, whereas in front-surface fluorometry, this angle is always acute (< 90°).

For the determination of ZP, a tungsten lamp, modified by an appropriate filter, provides a maximum excitation energy at ~ 420 nm. The light is focused on a glass microscope slide coverslip, upon which a blood drop forms an optically opaque specimen (Figure 4) Within the intact erythrocyte, ZP has a fluorescence maximum at 596 nm, while Hb strongly absorbs incident light at the excitation wave-length known as the Soret band. Fluorescence from ZP is detected by a red-sensitive photo-multiplier tube generating an analog signal, the intensity of which is proportional to the ZP/Hb molar ratio. The emission intensity can be calculated using the following formula:

$$I_{em} = \left(10^{-6} K I_{ex} \frac{M_{Hb}\ \varepsilon_{z}^{424}}{M_{z}\ \varepsilon_{Hb}^{424}} \right)$$

where:

I_{em} = the emission intensity

I_{ex} = the excitation intensity

C_{Hb} = the concentration of *Hb*

M_{Hb} = the relative molecular mass of *Hb*

C_z = the concentration of ZP

M_z = the relative molecular mass of ZP

K = the geometrical factor

ε = the molar absorptivity.

Any absorbance of incident light by ZP is considered negligible compared to *Hb*.

Also, the equation assumes that ZP is the only significant fluorophor. Because the emission intensity is proportional to the molar

ratio of ZP to Hb, the displayed result is independent of the volume of blood on the coverslip.

9.2 Materials and Reagents

Few materials are required for the operation of a HF.

- 25- x 25-mm microscope cover slips (#1 grade)
- A dispensing and stirring device (e.g., 25-µL micropipet with disposable tips)
- Blood-collection containers anticoagulated with EDTA (heparin and citrate are also acceptable anticoagulants)
- Control materials.

The following items are needed to operate all HFs:The following safety items are also recom-mended:

- Eye protection
- Disposable latex or vinyl gloves
- Protective face shield
- Biohazard waste bag contained within an appropriately labeled, puncture-proof box (use of a plastic biohazard bag alone is not recommended).

Due to differences in brands of HFs, additional materials can be required for the operation of certain hematofluorometry instruments:

- Derivatizing reagent
- Calibrator solutions (manufacturer supplied)
- 50-µL micropipet
- 10- x 75-mm glass tubes.

9.3 Specimen Requirements

Blood analyzed using the HF should be as fresh as possible. The HF was designed to be used in the field at screening sites where immediate results allow rapid response. Specimens collected at a remote site should be transported to the laboratory for analysis as soon as possible after collection. Blood specimens may be collected in evacuated tubes using venipuncture, or in various microcontainers using fingerstick techniques. EDTA and heparin are recommended anticoagulants, but citrate is also acceptable.

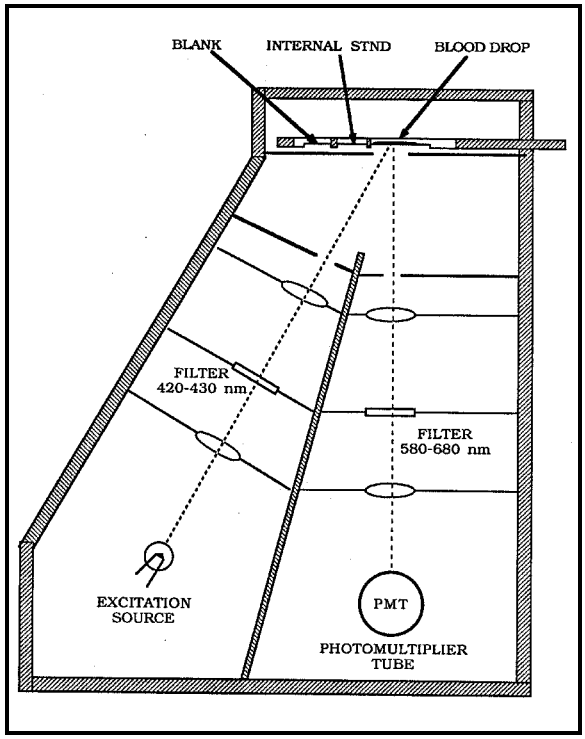


Figure 4. Optical design of the prototype hematofluorometer.

Transportation by mail at ambient temperatures is acceptable. However, if either mailing or analysis is delayed, blood specimens should be stored/refrigerated at 4 °C. Hemolysis adversely affects results, so blood analyzed by hemato-fluorometry should never be frozen. There is one report that blood samples stored for 1 month at 4 °C showed no adverse affects when analyzed using hematofluorometry.[65] However, the recommendations of instrument manufacturers and other data suggest that hematofluorometry values change after several days, even when blood samples are refrigerated at 4 °C.[66] Unlike analysis for EP by extraction, hemolysis and the gradual

formation of decomposition products can adversely affect results.

Many HFs require fresh, oxygenated blood, which is bright red in appearance. This required oxygenation of blood specimens is more difficult to attain when specimens are stored before analysis. Blood specimens that are hemolyzed, clotted, or visibly decomposed are unsuitable for analysis.

9.4 Instrument Calibration

The various brands of HFs differ somewhat with respect to calibration. Some instruments are factory calibrated and they are not designed for easy recalibration by operators. These instruments are often returned to the manufacturer when there are indications that recalibration is needed. The frequency of factory recalibration is largely dependent on the environment in which the HF is used. Generally, instruments located in centralized clinical laboratories maintain calibration longer than those that are used predominantly in a field setting and regularly transported from site to site.

In large clinical laboratories where the level of technical support is likely to be high, operator recalibration of these instruments can be achieved through the use of blood-based reference materials. A suitable procedure for operator calibration is discussed in Section 9.7.

Other instruments require regular calibration adjustment by the operator as a part of routine instrument operation. The procedure for calibration adjustment of these instruments is included in Section 9.6.

9.5 Operating Procedure: General

These operating procedures apply to all HFs, with the exception of some specific differences in the operation of instruments that require the use of a proprietary derivatizing reagent. This reagent should be used only with instruments that expressly require its use. Some unique aspects of operating these instruments are described separately in Section 9.6.

(1) Switch the instrument on, and run through 3 to 5 measurement cycles. This is sufficient to warm up the instrument.

(2) Use disposable gloves when handling human blood. Because opening glass tubes and transferring blood aliquots can result in the generation of aerosols, appropriate face protection should be used.

(3) All specimens and QC materials should be well mixed before analysis. Venous specimens can be mixed on a laboratory rotator or rocker. The best method for mixing fingerstick specimens depends on the type of microcontainer used. Specimens collected in microhematocrit capillary tubes should be dispensed into a suitable container (e.g., small tube or microtiter well) for mixing. Most other microcontainers can be vortexed gently to mix the contents.

(4) Check the instrument's calibration using blood-based controls or reference materials. To record performance data and other maintenance information, a log book should be established for each instrument.

(5) Place a single, clean microscope coverslip in the sample holder (coverslips tend to stick together). Grasp the coverslip by the edges to avoid contaminating the surface with oils or glove powder, which can falsely elevate readings. Be careful to avoid injury when separating coverslips.

(6) Preferably, blood should be collected in a suitable microcontainer or evacuated tube, and a 25- to 50-μL aliquot should be transferred to the coverslip. A plastic transfer pipet or micropipet is suitable for this purpose. However, it is also acceptable to transfer a blood droplet obtained using the fingerstick method directly onto the coverslip. To avoid accidental injury or loss of the specimen, care must be taken when using this procedure.

(7) The sample volume should be large enough to cover the hole in the sample holder. Use of too much blood can cause problems by contaminating the sample holder (see Section 9.9.4 for information on spill cleanup). The use of too little blood can result in sample desiccation, which causes erroneous results.

(8) On most models, the blood should be stirred to convert all the hemoglobin into oxyhemoglobin, which is required for reliable measurements. The plastic transfer or micropipet tip works well for stirring. Implements made of glass or other material that can scratch the coverslip should not be used for stirring. The use of wooden implements is not recommended because they have been reported to cause spuriously high readings.

(9) Before taking a measurement, use the stirring tool to move any air bubbles or small fibrin clots that might be present outside of the aperture in the sample carrier. After the initial measurement, the specimen might need to be stirred again and the measurement repeated. Continue this process until stable readings are obtained.

(10) Individual instruments can vary, depending on the manufacturer and/or model, as to the exact mechanism by which a reading is taken. The procedure involves sliding the sample holder into the instrument manually or by automated operation. Consult the instrument operation manual for more detailed instructions.

(11) Place all blood-contaminated materials and waste (coverslip, pipet tips, and wipes) in a clearly marked, puncture-proof, red biohazard container. Dispose of all medical waste in accordance with local statutes and regulations.

9.6 Operating Procedure: Instruments That Use a Derivatizing Reagent

Some instruments differ from others in that a reagent is added to the blood to eliminate the need for sample oxygenation. The reagent should be used only with those HF that expressly require it. Use of the reagent with other HF is reported to cause an elevation in results.[67] Also, manufacturer-supplied calibrators are used to adjust instrument calibration. Currently, these calibrator solutions contain ZP and hemin. They are provided with target values (1) in units of μmol ZP/mol of heme traceable to human blood analyzed by HPLC and multichannel hematology analyzers and (2) in units of μg EP/100 mL of whole blood, Hct 35 (or 42) traceable to human blood analyzed by the ethyl acetate–acetic acid extraction procedure.

(1) Switch the instrument on, and run through 3 to 5 measurement cycles. This will sufficiently warmup the instrument.

(2) When handling blood specimens, use disposable gloves. Because opening glass tubes and transferring blood aliquots can result in the generation of aerosols, appropriate face protection should be used.

(3) All specimens and QC materials must be well mixed before analysis. Venous specimens can be mixed on a laboratory rotator or rocker. The best method for mixing fingerstick specimens depends on the type of microcontainer used. To mix the contents, most micro-containers can be vortexed gently.

(4) Before each run, instrument response must be checked using calibrators provided by the manufacturer. Read the high-level calibrator, and make any necessary adjustments to the gain (calibration slope) according to the manufacturer's instructions. Then read the low-level calibrator to check calibration before proceeding with controls (see Section 9.9.1) and patient samples.

Note that the derivatizing reagent is not used with the calibrator solutions. Performance data and other maintenance information should be logged for each instrument.

(5) Place a single, clean microscope cover-slip in the sample holder (coverslips tend to stick together). Carefully grasp the coverslip by the edges to avoid contaminating the surface with oils or glove powder, which can falsely elevate readings.

(6) Patient blood does not need to be oxygenated before taking a reading. Instead, an aliquot of well-mixed blood should be added to the derivatizing reagent in a 1:2 quantitative ratio. The solution should then be mixed gently before pouring it onto the coverslip for measurement. The sample volume should be large enough to cover the hole in the sample holder. Excess sample can cause problems in measurement accuracy by contaminating the sample holder (see Section 9.9.4 for information on spill clean-up). A con-venient volume to use is 25-μL blood plus 50 μL of reagent. If multiple measurements of a sample are desired, these volumes can be multiplied, and separate aliquots can be removed and measured. The mixture should be assayed as soon as possible after preparation.

(7) Because of photodecomposition, once an aliquot of prepared sample is placed on a coverslip, only one valid measurement can be taken. When multiple measurements of a sample are desired, fresh aliquots of blood/reagent mixture must be used. Any air bubbles or small fibrin clots that might be present should be moved outside of the hole in the sample carrier.

(8) Place all blood-contaminated materials and waste (coverslip, pipet tips, and wipes) in a clearly marked, puncture-proof, red biohazard container. Dispose of all medical waste in accordance with local statutes and regulations.

9.7 Results

For the following reasons, the subcommittee recommends the use of μmol/mol reporting units in hematofluorometry:

- These units represent a more accurate indication of protoporphyrin status[68-70]
- Inherent errors associated with the use of assumed Hb or Hct are avoided.

However, the units used in the field to report results obtained by hematofluorometry vary widely. Users should check each instrument carefully to determine the reporting units.

9.7.1 Micrograms "Equivalent" Erythrocyte Protoporphyrin (EP) per Deciliter of Whole Blood (μg EP/dL)

The most common units currently in routine use are micrograms of EP per deciliter (100 mL) of whole blood (μg EP/dL). For instruments that report concentration units based on whole blood, a specific Hb concentration, or Hct value, must be assumed. Most instruments in the field express the assumed value in terms of Hct. For pediatric populations, the assumed Hct value is 35%; for adult populations it is 42%. This assumption introduces a small amount of error into the result whenever the true sample Hb or Hct deviates from the assumed value.

These errors do not significantly affect the diagnostic utility of the result, and, in cases where Hb and/or Hct levels are depressed, the sensitivity of the test may actually be enhanced. Therefore, routine correction of hematofluorometry results is not recommended. However, if the true sample Hct is known, the hematofluorometry result can be corrected by multiplying by the following conversion factor:

$$EP\ Result\ \mu g/dL \cdot \frac{Sample\ Hematocrit}{Assumed\ Hematocrit} = Hct\ Corrected\ EP\ Result$$

For screening purposes, the reference interval, based on a value of 297 $L \cdot mmol^{-1} \cdot cm^{-1}$ for the absorptivity of PPIX, has an upper limit of 30 μg/dL (0.53 μmol/L) (see Section 10).

9.7.2 Micromoles ZP per Mole of Heme (μmol ZP/mol Heme)

This reporting unit is becoming increasingly accepted, and it is the subcommittee's recommendation that it be adopted universally. It is based on the ZP/heme molar ratio, which is independent of blood volume and, therefore, independent of the patient's Hct. Also, the ZP/heme molar ratio reflects more closely the physical measurement made by the HF. It is reported that the ZP/heme molar ratio reflects more accurately the early effects on the heme biosynthetic pathway that result from an iron-deficient state.[5] For screening pediatric populations, the upper limit of the reference interval, based on an empirical comparison with extraction EP data in μg/dL, is 70 μmol/mol.[71] Reference intervals are discussed in detail in Section 10.

9.7.3 Other Reporting Units

Though rarely encountered, instruments exist that have been calibrated in micrograms ZP per deciliter of RBCs (μg ZP/dL RBC), micrograms EP per gram hemoglobin (μg EP/g Hb), and possibly other units. The procedure used by manufacturers to calibrate these instruments is believed to be based on the application of mathematical conversion factors to blood specimens analyzed by extraction for EP and by multichannel hematology analyzer for Hb or Hct.

9.8 Interferences

A number of substances found in plasma are known to interfere in the determination of EP by hematofluorometry; sometimes, these substances can cause substantial elevations of EP results.[72] Bilirubin can interfere because its fluorescence maximum is approximately 525 nm.[73] This interference from bilirubin fluorescence can be more of a problem when using a hematofluorometer with an emission filter that permits fluorescence below 590 nm to reach the detector.[74]

Several drugs, including doxorubicin, spironolactone, amoxycillin, iron succinate, methotrexate, and doxycycline have been shown to increase hematofluorometry results.[75] Riboflavin has been reported to increase hematofluorometry results,[76] as has valproic acid.[16] Also, other as yet unidentified plasma artifacts can contribute to fluorescent intensity at the emission wavelength.[72]

Spurious fluorescence from plasma components can be eliminated by removing the plasma after centrifugation and then washing the erythrocytes with isotonic saline before measurement. Some investigators have advocated the use of such a washing procedure on a routine basis[72,75] and the washing procedure has the additional benefit of resulting in complete specimen oxygenation (unpublished data, Wisconsin State Laboratory of Hygiene).

However, the subcommittee does not recommend the incorporation of a washing procedure into the routine operation of HF. The influence of plasma fluorescence is uniformly positive and, in healthy populations, is moderate and relatively constant.[75] Whole blood results above the reference range, whether real or due to plasma fluorescence, constitute a distinct minority in most tested populations. The speed and simplicity of analysis are major attributes of hematofluorometry. For these reasons, the added complexity of an erythrocyte washing procedure is not considered justified. In addition, microspecimens collected using the fingerstick method, which is most commonly used in the population, can have inadequate volume to perform such a procedure.

For whole blood results that are above the reference interval, a washing procedure is useful for the elimination of false-positive results caused by fluorescing plasma constituents. An appropriate washing procedure is described by Hastka et al.[75]

9.9 Quality Assurance/Quality Control (QA/QC)

When HFs were first developed, QA/QC was accomplished by taking a series of readings using "checker" slides provided by the manufacturer. The checker slides contained fluorescent compounds, such as Rhodamine B or synthetic polymers, that had spectral properties similar to those of ZP. Since the early 1980s, checker slides are no longer recommended by manufacturers, largely because they were found to be inadequate

and unreliable. Nonetheless, a robust QA/QC procedure is desirable, and it is preferable that this be independent of the factory calibration.

9.9.1 Reference Materials for Use with Hematofluorometers

A number of blood-based materials have been developed in recent years for use as QC products. Some of these materials have been produced by instrument manufacturers and have a role in factory calibration (described in Section 9.9.2). Such materials can be considered calibration checks and are useful as such, but they might not be truly independent of the original factory calibration. Although the number of suppliers is limited, it is recommended that users of a HF obtain control or calibrator materials from at least two different sources.

The composition and characteristics of these materials vary. An interlaboratory study indicates that the quality of these materials differs with respect to stability and target value assignment.[16] While improvements have been made since this study was completed, it is important to note that the suitability of specific reference materials can differ for the various brands of HFs that are available.

9.9.2 Recalibration of Hematofluorometers

As the use of μmol/mol reporting units gains wider acceptance, users might wish to convert their instruments so that they are capable of reporting in these units. Such a conversion is possible in most instruments, but it is not clear whether the necessary adjustments can be made by users. The manufacturer should be consulted for more information.

Regardless of the reporting units used, instrumental drift can occur, for a variety of reasons, with all brands of HFs; this can result in an out-of-calibration condition. For those users without sufficient technical knowledge, the only recourse is to return the HF to the manufacturer for recalibration. In some cases, factory-based calibration involves comparing digital readings between a client's instrument and a "master" instrument.

Calibration of the master instrument is maintained by using blood-based reference materials that are traceable to some reference method. In other cases, calibration at the factory is performed using reference materials.

Users can recalibrate HF in house using reference materials or a direct comparison of patient results to the reference method (usually extraction) results. Direct comparison is considered to be the best method of calibration for HFs, but it is impractical for most laboratories. A large number of suitable blood specimens must be run in parallel on the HF using a reference method, and the resulting data should be examined by linear regression analysis. Because of the scatter observed in hematofluorometry/extraction comparisons, this usually requires a sample size $\geq$ 40, with results covering the commonly encountered range of values.

Recalibration can be performed relatively simply using blood-based reference materials and a few simple tools. A suitable procedure is described below.

NOTE: This procedure is appropriate for most instruments, with accessible zero and gain adjustments. Certain instruments can require a different procedure.

The following equipment is necessary:

- Small flat-blade screwdriver (e.g., a jeweler's screwdriver)
- At least three blood-based reference materials with defined target values, encompassing normal and elevated values.
- Access to an instrument for performing simple linear regression (LR) analysis. Hand-held calculators with a LR function or statistical programs available for personal computers are appropriate to evaluate the slope and intercept parameters.

The procedure is as follows:

(1) If the HF reports results in units that require an assumed Hct, ensure that the Hct corresponds to the target values assigned to the reference materials. For example, if the materials have values that assume a fixed Hct

of 35, and the HF is designed to assume a fixed Hct of 42, target values must be multiplied by 1.2 to obtain the corrected target values at Hct 42.

(2) Analyze the control/reference materials in the same manner as patient specimens. Record the values and plot the results as a function of the target value, i.e., target value on the *x* axis, HF result on the *y* axis.

(3) Find the straight line that best fits the data using least squares regression analysis. Calculate the slope and intercept.

(4) Examination of the plot can indicate that one or more of the materials is an outlier. If a sufficient number and range of reference values remain, the outlier can be rejected and the regression parameters recalculated. Alternatively, a different reference sample may be substituted. If the reference data continue to show anomalies, consult the manufacturer.

(5) Recalibration may be indicated if either the slope ≠ 1 and/or the *y*-intercept ≠ 0. In practice, a slope that is in the range 0.95–1.05 and a *y*-intercept of ±3 is acceptable. Further optimization, although possible, can be difficult to achieve.

(6) Calibration correction is achieved by adjusting the zero (*y*-intercept) and/or calibration or gain (slope) potentiometers ("pots"). The location of these potentiometers varies among manufacturers and instrument models. Consult the operation manual or manufacturer if one is unsure about the location of the potentiometers.

(7) Begin by making an adjustment to the instrument zero. Use a low-concentration reference material for this step and take repeated readings while adjusting the zero pot, until the indicated value has been changed by the same amount, and in the opposite direction, as the calculated *y*-intercept.

(8) Make any appropriate adjustments to the calibration (or gain) pot as indicated by the slope value, using a high-concentration reference material.

(9) Recheck calibration by measuring the reference materials again and replotting the data, as described in steps 2 and 3.

(10) If necessary, repeat steps 7 through 9 to make any additional fine adjustments to the calibration.

(11) Further QA checks on the calibration can be performed using a control/reference material from a different source. There may be a small bias between materials from different sources, but the measured values should be within the acceptable range.

9.9.3 External QA

In addition to the internal QA procedures outlined above, it is highly desirable, and it can be required by regulatory authorities, that hematofluorometry users participate in an external quality assessment or proficiency testing program. Participation in such programs can be used to monitor performance and identify trends before a problem becomes serious. Such programs also help to promote the standardization of laboratory data. Additional information on external QA can be found in Appendix B.

9.9.4 Preventive Maintenance

HFs require little preventive maintenance. It is preferable to keep the HF in one location. HFs used in several different sites should be transported in a suitable container designed to protect the equipment. The HF should not be exposed to extreme temperatures. If it is exposed to cold temperatures, always allow 30 minutes for the HF to return to room temperature. Sample holders that are contaminated with blood should be cleaned with a 5% (v/v) bleach solution and rinsed with deionized water. Do not use organic solvents, detergents, or other cleaning fluids that can contain fluorescing compounds. Do not use

abrasives on the sample holder, because some instruments are designed to take a dark current reading from the underside of the holder.

10 Interpretation of Erythrocyte Protoporphyrin Values

10.1 Erythrocyte Protoporphyrin Reporting Units

The use of multiple different reporting units for EP has been a source of confusion and a deterrent to the wider use of the test. With the exception of units expressed on the basis of whole blood, all units for EP are based on a RBC, or their heme or hemoglobin constituents. The reason for using a heme-, hemoglobin-, or RBC-based unit is that virtually all of the protoporphyrin in the blood is contained within the RBCs.

Furthermore, expressing EP on the basis of heme or hemoglobin recognizes a precursor–product relationship, because protoporphyrin plus iron combine to form the heme portion of hemoglobin. The subcommittee recommends the use of μmol EP/mol heme as the universal reporting unit because this ratio expresses a precursor–product relationship and it can be measured directly by a HF. When the extraction method is used to determine EP, it is also necessary to determine the concentration of hemoglobin in order to express the results as μmol/mol heme. Generally, this is possible because, in most circumstances, EP screening is combined with screening for anemia. Thus, the preferred unit is: μmol/mol of heme.

The other three units currently in use are:

- μg/dL of whole blood
- μg/dL of RBC
- μg/g of hemoglobin.

The whole blood unit came into wide use because it was recommended by the CDC as the reporting unit for CDC-supported childhood lead poisoning screening programs. Then, most HF were configured to report results in this fashion.[24] The main advantage of the whole blood unit is that the EP results do not require the measurement of Hb or Hct. However, a major disadvantage is that it is less accurate in characterizing the disturbance in heme production because it relies on assumed, rather than measured, Hb or Hct values. Probably, this was not a serious source of error before 1985 when lead poisoning was defined on the basis of a blood lead ≥ 30 μg/dL (1.45 μmol/L), because corresponding elevations in EP were likely to be well above the upper threshold of the reference interval. However, using a molar ratio more accurately reflects disturbances of heme synthesis, in addition to avoiding these inherent errors.

10.2 Reference Intervals

Because low EP values have no known clinical significance, the focus of interest is on the upper limit of the reference interval. Based on the method of Piomelli,[77] the most commonly used EP threshold value for childhood lead screening has been 35 μg/dL (0.62 μmol/L) of whole blood. Because the millmolar absorptivity for EP in the Piomelli method was 241 $L \cdot mmol^{-1} \cdot cm^{-1}$, the equivalent cut-off value for the NCCLS-recommended method, based on an $m\varepsilon$ of 297 $L \cdot mmol^{-1} \cdot cm^{-1}$, is 28 μg/dL (0.50 μmol/L) of whole blood. Table 2 lists the median, 90%, and 95% range of EP values from NHANES II, after excluding persons with anemia, low transferrin saturation, and elevated blood lead.[78]

The recommended threshold values tabulated below lie between the 95th percentile value for children and for women. The reason for favoring the 95th percentile value, rather than the 97.5 percentile value, is that EP is used as a screening test, which requires further evaluation, and not as a diagnostic test. Thus, a lower threshold value gives a greater benefit of doubt to define abnormal results.

Conversion of Reporting Units:

The following conversion factors are based on the molecular weights of protoporphyrin and Hgb and the molar ratio of Hgb/heme. Further details about the derivation of conversion factors 1 and 2 are addressed in reference 71.

(1)

$$\frac{\mu g\ EP}{100\ mL\ Blood} \bullet \frac{100\ mL\ Blood}{Sample\ Hct\ (\%)} \bullet 80 = \frac{\mu mol\ EP}{mol\ Heme}$$

(2)

$$\frac{\mu g\ EP}{100\ mL\ Blood} \bullet \frac{100\ mL\ Blood}{Sample\ Hb\ (g)} \bullet 25.8 = \frac{\mu mol\ EP}{mol\ Heme}$$

(3) $$\frac{\mu mol\ EP}{L\ RBC} \cdot 50 = \frac{\mu mol\ EP}{mol\ Heme}$$

The derivation of conversion 3 is as follows:

Hct (%)/3.1 = Hgb (g/dL)
Hct (%)/0.31 = Hgb (g/L)
L RBCs = Hct 100% so L RBCs · 100/0.31
= 323 g Hgb.

$$\frac{\mu mol\ EP}{L\ RBCs} \cdot \frac{L\ RBCs}{323\ g\ Hgb} \cdot \frac{64458\ g\ Hgb}{1\ mol\ Hgb} \cdot \frac{1\ mol\ Hgb}{4\ mol\ heme}$$

$$= \frac{\mu mol\ EP}{mol\ heme}$$

Reduced, this yields:

$$\frac{\mu\ mol\ \ EP}{L\ RBC} \bullet 50 = \frac{\mu\ mol\ \ EP}{mol\ \ Heme}$$

It is convenient to use the same threshold values for men who are being evaluated for industrial lead exposure despite the fact that their 95th-percentile value is substantially lower than those for women and children (Table 2). This can be justified by the relatively high threshold concentration of lead specified by OSHA guidelines and the expected significant elevations of EP associated with those lead concentrations.[79,80] The same threshold values have also been used in combination with Hb or Hct to screen blood donors for iron deficiency.[31] Recommended upper threshold values of the reference range for EP are as follows:

(1)	μmol/mol of heme	70
(2)	μg/dL whole blood	30***
(3)	μg/dL RBC	80
(4)	μg/g hemoglobin	2.5.

Note that any change to the recommended reference interval must be clearly communicated to the practicing clinician to prevent incorrect interpretation of EP results.

10.3 Interpretation of Erythrocyte Protoporphyrin Results

10.3.1 Iron Deficiency and Lead Poisoning

The EP test is most commonly used in screening for iron deficiency and lead poisoning. Because both of these conditions often coexist among children,[24,81-83] an elevated EP value often is the result of both conditions.[81] The probable basis for this association is that iron deficiency can predispose one to lead poisoning by enhancing lead absorption.[83] Therefore, in the evaluation of children with an abnormal EP, the finding of elevated blood lead by no means excludes the possibility of iron deficiency, and it may indeed support that diagnosis. Because iron deficiency is far more common than lead poisoning, most elevated EP values in children are due to iron deficiency.

10.3.2 Iron Deficiency and Infection

Both iron deficiency and mild infection are common in early childhood and they cause mild to moderate elevation of EP.[41] The presence of recent or current illness can complicate the screening for lead poisoning or iron deficiency with the EP test (see Section 6).

10.3.3 Hemolytic Anemia or Increased RBC Production

A significant increase in RBC production, regardless of the reason, can result in an elevated EP concentration. Consequently, in persons with sickle cell anemia (not sickle cell trait), tests other than EP should be used to diagnose lead poisoning or iron deficiency.

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*†Note: This value differs slightly from the threshold value of 28 μg/dL (0.50 μmol/L) at $m\epsilon$ 241 L · mmol^{-1} · cm^{-1} to the corresponding value using an $m\epsilon$ of 297 L · mmol^{-1} · cm^{-1}.

Table 2. EP Concentration Determined by Extraction for Children 1–5 Years of Age, Women 18–44 Years, and Men 18–44 Years After Excluding Persons with Anemia, Iron Deficiency, and Elevated Blood Lead

Percentile:	2.5th	5.0th	50th	95th	97.5th
μmol/mol of Heme					
Children	28.4	30.6	45.5	72.5	83.3
Women	28.6	31.2	43.2	65.3	75.0
Men	26.2	27.8	37.5	53.0	57.0
μg/dL Whole Blood					
Children	12.5	13.4	19.5	30.8	35.0
Women	13.7	14.8	20.4	30.3	33.1
Men	13.9	14.8	20.2	28.3	31.1
μg/dL RBC					
Children	34	37	54	85	96
Women	34	37	51	76	87
Men	31	33	45	64	68
μg/g Hb					
Children	0.99	1.06	1.59	2.52	2.87
Women	1.00	1.09	1.51	2.28	2.62
Men	0.92	0.97	1.31	1.85	2.00

Data derived from NHANES II.

Appendix A: Preparation of Quality Control Materials for Extraction Methods

A.1 Normal Human Concentration Materials

(1) Using standard American Association of Blood Banks-approved methods, collect whole blood from normal, healthy HIV- and hepatitis B-negative subjects into 250-mL vacuum collection bottles with 10 μL of 15 g/dL K_3EDTA per mL blood added, or in standard 450-mL blood collection bags with EDTA solution added. Obtain the Hb or Hct result for concentration correction if desired, and use this result for the duration of the pool's use.

(2) If a large volume is required, mix the contents of the bottle(s) well, and combine into a 1-L flask that was previously rinsed with 0.1 mol/L HCl, followed by deionized water, then dried.

(3) Dispense 0.5 mL aliquots into sealable 1.0-mL glass vials or high-density polypropylene vials with multi-turn caps.

(4) Store the prepared vials at ≤ -70 °C. Materials are stable for over 3 years at this temperature, especially if stored in sealed glass vials.[52]

(5) Lyophilization may be used to preserve materials. Store lyophilized materials at ≤ -20 °C for maximum stability.

A.2 Elevated Concentration Materials

Using procedures approved by an appropriate animal welfare review committee, obtain elevated PPIX levels in blood by "dosing" large, healthy animals, such as cows or goats. An undosed animal will have a normal PPIX level, which is adequate for a mid-range elevated pool. Moderate dosing provides a high concentration pool. Depending on the species, it can take several days to several months to produce elevated concentrations. Baseline and several successive specimens may need to be collected from a new animal to verify response to the lead dose before making a final large-volume blood collection. (The following instructions assume the test animal is a cow).

(1) Obtain the current weight of the animal.

(2) Prepare a dose containing 3 g of lead nitrate in a size-12 gelatin capsule.

(3) Administer the dose to the animal.

(4) Collect 1 L of EDTA-anticoagulated blood from the cow 48 hours after dosing, using appropriate sterile technique.

(5) Check the PPIX concentration of the blood collected from each animal by removing an aliquot of blood with sterile technique, then assaying.

(6) Obtain the desired concentration levels by mixing different units with blood from an undosed animal, if needed.

(7) Dispense and store blood as described in Section A.1(4) above.

Appendix B: Proficiency Testing and Laboratory Certification

Consistent with general QA practices in the clinical laboratory, any laboratory that performs EP determinations should participate in proficiency testing (PT) or interlaboratory comparison programs. Participation in these programs provides an external evaluation of performance and allows the identification and correction of problems that may not be apparent from in house QC practices. In many cases, PT programs serve as a source of current information and promote the standardization of EP measurements in the field.

Several such programs are available currently, but some have enrollment restrictions and/or certification functions. Following is a list of currently available programs:

- *Sponsoring Agency*: Cooperative effort of the Maternal and Child Health Bureau and the Wisconsin State Laboratory of Hygiene (MCHB/SLH).

  Scope: Open to any laboratory.

  Frequency and Number of Challenges: Specimens provided monthly. Three specimens per shipment.

  Licensing/Certification Function: None. Data may be used by individual states for regulatory purposes.

  Additional Comments: For more information contact the Toxicology Section, State Laboratory of Hygiene, 465 Henry Mall, Madison, WI 53706. Telephone 608.262.1146.

- *Sponsoring Agency*: New York State Department of Health (NYSDOH).

  Scope: Participation required by laboratories analyzing specimens from New York residents. The program is open to other laboratories.

  Frequency and Number of Challenges: Specimens provided three times yearly. Five specimens per shipment.

  Licensing/Certification Function: Program data are used to license laboratories analyzing specimens from New York residents. Data may be used by other states for regulatory purposes.

  Additional Comments: For more information, contact the New York State Department of Health, Wadsworth Center for Laboratories and Research, Empire State Plaza, Albany, New York 12201. Telephone 518.474.5475. Separate programs exist for laboratories using extraction methods and hematofluorometers.

- *Sponsoring Agency*: Pennsylvania Department of Health.

  Scope: Participation required by and restricted to laboratories in or serving Pennsylvania residents.

  Frequency and Number of Challenges: Specimens provided three times yearly. Five specimens per shipment.

  Licensing/Certification Function: Program data used to license laboratories analyzing specimens from Pennsylvania residents.

  Additional Comments: For more information, contact the Pennsylvania Department of Health, Bureau of Laboratories, Pickering Way and Welsh Pool Road, Lionville, Pennsylvania 19353.

Telephone 610.363.8500. Currently, no provision has been made for evaluating laboratories reporting results in SI units.

- *Sponsoring Agency*: New Jersey State Department of Health (NJDOH).

  Scope: Participation required by and restricted to laboratories serving New Jersey residents.

  Frequency and Number of Challenges: Specimens provided three times yearly. Five specimens per shipment.

  Licensing/Certification Function: Program data used to license laboratories analyzing specimens from New Jersey residents.

  Additional Comments: For more information, contact the New Jersey State Department of Health, Clinical Laboratory Improvement Program, CN 360, Trenton, NJ 08625. Telephone 609.530.6172.

Laboratories that perform the analysis of EP are subject to the general provisions of the Clinical Laboratory Improvement Amendments (CLIA '88; 42 CFR 493). Although PT requirements for EP are not specified, laboratories should comply with the appropriate requirements for patient test management, QC, personnel standards, and other aspects of the regulations. Laboratories should become familiar with these federal regulations as they relate to EP testing before reporting patient results.

Several individual states regulate or license laboratories that perform EP testing. Depending on the location of the laboratory and the specimen base, participation in one or more PT programs may be required. The licensing/certification functions of state-sponsored PT programs are noted in the preceding table. At least one additional state evaluates data from other PT programs to monitor the performance of laboratories in that state. Therefore, laboratories should also become familiar with requirements of the state(s) that comprise their EP specimen base before reporting patient results.

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Summary of Comments and Subcommittee Responses

C42-P: *Erythrocyte Protoporphyrin Testing; Proposed Guideline*

General Comments

1. The recommendation to use erythrocyte protoporphyrin (EP) when using acid extraction to liberate zinc and measure all nonheme erythrocyte protoporphyrin in the free form is good. Our laboratory presently reports as total erythrocyte protoporphyrin (TEP) and reserves free erythrocyte protoporphyrin (FEP) for the native free erythrocyte protoporphyrin. However, some laboratories report results from the acid extraction method as FEP because the protoporphyrin is measured in the free form.

- **The terminology and abbreviations associated with this analysis have long been a source of confusion. The subcommittee is pleased to see support for the terminology recommended in this guideline.**

2. Inclusion of a method for the preparation of normal and elevated pools for quality control is good. *The availability of commercial controls is mentioned. We recommend including manufacturers names so this option can be investigated.*

- **Provision of a list of available control products was considered by the subcommittee. It was ultimately rejected due to concerns about inclusiveness and strong discouragement of the use of commercial names by NCCLS. A list of control products can be obtained through the subcommittee chairholder (Noel V. Stanton, MS, University of Wisconsin, WI State Laboratory of Hygiene, 465 Henry Mall, Madison, Wisconsin 53706; phone: 608.262.1146; fax: 608.262.5494) as a service of the Federal EP Proficiency Testing Program.**

3. The guideline offers good recommendation to adopt a specific molar absorptivity constant (ME) for standards. I was not aware of the significant discrepancies in standards.

   The standard used in our laboratory is based on an ME of 241 L $mmol^{-1}$ cm^{-1}, not the recommended 297 L $mmol^{-1}$ cm^{-1}. A conversion factor of 241/297 can be applied. This factor will also need to be applied to our reference range.

- **It is gratifying to see that information in the guideline is supported by observations in the field.**

4. As a result of reading C42-P, our laboratory picked up some suggestions that might improve our results. These include:

   - Molecular concentration should be calculated for each lot number of HCl

   - Ethyl acetate/acetic acid should be prepared fresh weekly and stored in an amber bottle. Presently our laboratory prepares this reagent every 3 months.

   - Our laboratory has had problems with ethyl acetate; so, it was interesting to note that ethyl acetate is the most frequent source of error and to learn about the KI test to test the reagent for quenching agents.

- **It is gratifying to see that information in the guideline is supported by observations in the field.**

5. Our laboratory also determined that EDTA is the best anticoagulant and that samples are stable 2 weeks at 4 °C. We also noted a loss of linearity at high concentration and dilute

accordingly, but it was interesting to note that it might be due to fluorescence quenching, not extraction efficiency.

- **It is gratifying to see that information in the guideline is supported by observations in the field.**

6. The discussion of previously incorrect assignment of the molar absorptivity of protoporphyrin IX was interesting. The recommendation to standardize all methods to a single value and reassignment of reference ranges seems appropriate. However, exactly how to communicate a change in the assay standardization to the practicing clinician was less well described. Because reporting erythrocyte protoporphyrin in the "new" units might represent as much as a 19% change from the "old" units a clinician is used to seeing, some recommendation to notify clinicians that the unit has changed might be wise. In my experience many clinicians do not look at the reference range closely, and merely changing the reference range might not be adequate notification of a calibration change.

- **Effective communication to clinicians of changes to the reference interval is an important point. Wording was incorporated into Sections 7 and 10.2 that stresses the importance of communicating such changes.**

  The subcommittee plans to make clinicians aware of this issue via a letter submitted to appropriate journals, communications to participants in various EP proficiency testing programs, and information distributed by relevant manufacturers. A firm timetable for implementation of this plan has not been established.

Section 8.9

7. *We would like to see HPLC methods discussed in detail like other methods*. This is the method used in our laboratory. If you have an HPLC system, it is not a difficult procedure for a routine laboratory. Our laboratory uses acid extraction to measure total erythrocyte protoporphyrin or dimethylformamide extraction to measure free and zincprotoporphyrin separately.

- **As noted in C42-A, Section 8.9, the opinion of the subcommittee is that HPLC methods are employed primarily for the differential quantitation of several porphyrins, rather than for EP specifically. A notable lack of EP proficiency testing participants using HPLC methods indicates that these methods are not widely used for EP testing. Consequently, a more thorough treatment of HPLC methods was not incorporated into the document at this time. The option remains open for the future, pending additional input on the subject.**

Section 10.1

8. The recommendation for the universal adoption of reporting units is good. Our laboratory presently reports as μmol/L RBCs. *We recommend including conversion of μmol/L RBC to μmol/mole heme in the conversion of reporting units section, along with a better explanation of conversions. In the examples given, the numbers used (e.g., 80 and 25.8) are not defined.*

- **The requested conversion from μmol/L RBC to μmol/mol heme was incorporated into C42-A. The conversion was derived using the following calculations:**

Hct (%)/3.1 = Hgb (g/dL)
Hct (%)/0.31 = Hgb (g/L)
L RBCs = Hct 100%, so L RBCs · 100/0.31 = 323 g Hgb

$$\frac{m\ \text{mol EP}}{\text{L RBCs}} \bullet \frac{\text{L RBCs}}{323\ \text{g Hgb}} \bullet \frac{64458\ \text{g Hgb}}{\text{mol Hgb}} \bullet \frac{1\ \text{mol Hgb}}{4\ \text{mol Heme}} = \frac{m\ \text{mol EP}}{\text{mol Heme}}$$

Reduced, this yields:

$$\frac{m\ \text{mol EP}}{\text{L RBCs}} \bullet 50 = \frac{m\ \text{mol EP}}{\text{mol Heme}}$$

To address concerns about the basis for the included conversion factors, wording was added to Section 10.2 that notes that the conversion factors provided are based on the molecular weights of protoporphyrin and Hgb, the molar ratio of Hgb/heme, and that the derivation of the conversion factors is described in detail elsewhere. Reference 71 was moved to this location in the text, rather than preceding the conversions.

Related NCCLS Publications**

C28-A **How to Define and Determine Reference Intervals in the Clinical Laboratory; Approved Guideline (1995).** C28-A gives a protocol for the determination of reference intervals for defined populations to help with interpretation of laboratory data.

I17-P **Protection of Laboratory Workers from Instrument Biohazards; Proposed Guideline (1991).** I17-P offers guidelines for the protection of those who use and maintain laboratory equipment from infectious diseases transmitted in human body fluids and tissue that might contaminate equipment.

M29-T2 **Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, and Tissue—Second Edition; Tentative Guideline (1991).** M29-T2 provides guidance on the risk of transmission of hepatitis B virus and the human immunodeficiency virus in the laboratory. Specific precautions for preventing the transmission of bloodborne infection during clinical anatomical laboratory procedures are addressed.

**Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.