can use simple ultraviolet detection. Moreover, because our PCR products can be loaded directly onto the capillary, no preparative steps such as overnight digestion, precipitation, desalting, or extensive washing of the samples are required. This greatly speeds up the analysis and reduces both the labor and cost. Thus, our technique is ideally suited to semi-automation, and it can readily be applied to high-volume genotyping or screening programs.

This study was supported in part by a grant from the Dublin Institute of Technology, Ireland. We thank Dr. Kimmo Kontula and Dr. Ariella Zivelin for generously providing the *Apo E* reference samples.

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Observations on the Zinc Protoporphyrin/Heme Ratio in Whole Blood, *Robert F. Labbé,[1*] Anjana Dewanji,[2] Kathleen McLaughlin[1]* ([1] Department of Laboratory Medicine, Box 359743, Harborview Medical Center, Seattle WA 98104-2499, and [2] Biological Sciences Division, Indian Statistical Institute, Calcutta, 700 035 India; * author for correspondence: fax 206-731-3930, e-mail boblabbe@ix.netcom.com)

Zinc protoporphyrin (ZPP) is a normal metabolite that accumulates at trace amounts in erythrocytes during hemoglobin synthesis. In states of inadequate iron delivery to developing red cells, excess ZPP forms as a by-product of the heme biosynthetic pathway. This ZPP response occurs because iron and zinc interact as ferrochelatase substrates, with zinc utilization increasing when the iron supply is diminished *(1)*. The resulting high zinc protoporphyrin/heme ratio (ZPP/H) in circulating erythrocytes reflects a state of relative iron-deficient erythropoiesis *(2)*. Either the whole blood or erythrocyte ZPP/H ratio in μmol/mol can be determined conveniently using hematofluorometry *(3)*, a technique for front-surface fluorescence/absorption measurement *(4)*.

Despite its ease of determination and its clinical utility *(5)*, ZPP/H has found only limited application in patient care. Criticism of ZPP/H determination has been based largely on interference from plasma components, primarily bilirubin *(6–8)*. Other less common analytical pitfalls that have been cited include incomplete hemoglobin oxygenation *(9)* and the degree of hemolysis *(10)*. Accordingly, we undertook an evaluation of the analytical procedure to try to understand and alleviate these factors associated with hematofluorometry. When the ZPP/H ratio is determined as prescribed, we believe that clinically acceptable analytical quality can be maintained. Furthermore, the highly sensitive, cost-effective ZPP/H ratio has merit as a primary test for assessing iron status and for monitoring iron therapy.

For this evaluation, we used anticoagulated whole blood specimens obtained from our clinical hematology laboratories after all ordered tests had been completed. The blood had been collected in Vacutainer 3.0-mL Hemogard Tubes containing 3.0 mg EDTA (K3). Approval was obtained from the Human Subjects Division, Office of Research for the use of these leftover specimens without patient identification. Blood specimens were used either as collected or were processed as described for particular experiments.

Blood specimens were selected to have a ZPP/H ratio within our reference range (30–80 μmol ZPP/mol heme). For experiments to observe the effects of hemolysis, erythrocytes were washed twice with a 9 g/L sodium chloride solution to remove any plasma interference. We hemolyzed the washed cells pooled from several blood specimens, using one of three different methods: (*a*) suspending cells in distilled water and then reconstituting them with sodium chloride to a physiologic concentration of 9 g/L; (*b*) suspending cells in physiological saline and then subjecting them to freeze-thaw cycles; or (*c*) suspending cells in physiological saline containing 40 g/L Triton X-100. Erythrocytes hemolyzed by the first two methods could then be mixed in a series of proportions with washed cells to determine the effect of increasing degrees of hemolysis. Cells hemolyzed by the third method were used only as hemolyzed specimens in separate experiments.

The ProtoFluor-Z reagent and the two different hematofluorometers used were provided by Helena Laboratories. The Ultimate-D Bilirubin Control (Beckman Instruments), a serum-based preparation, was used as a source of exogenous bilirubin. This Bilirubin Control solution was added in various proportions to whole blood to produce a series of bilirubin concentrations similar to those found in clinical practice. The nonionic surfactant used to hemolyze cells was Triton X-100 purchased from Sigma Chemical Co.

Hemolyzed and whole erythrocytes were mixed in a range of 11 different proportions from 0% to 100% hemolysis. In a typical test series, these mixtures yielded essentially identical ZPP/H results regardless of the method or degree of hemolysis (mean ± SD, 36.2 ± 2.5 μmol/mol; range, 31–41 μmol/mol; CV = 7.7%). The CV for a typical hemolysis series was nearly twofold higher than the within-run CV of 4% experienced in this laboratory. This absence of an hemolysis effect was reproduced by several technologists using different blood specimens and different instruments. In every case, qualitatively comparable results were obtained. This was surprising yet not totally unexpected, because in a porphyrin fluorescence study that predated the hematofluorometer *(11)*, erythrocytes were hemolyzed with distilled water, and Triton X-100 was used as a solubilizing agent to produce favorable spectra, similar to the conditions described here.

In studying the effects of additives for ZPP/H determination (Table 1), we did not always strictly adhere to the instrument manufacturer's volume recommendations because the ZPP/H ratio is not influenced by dilution except in extreme cases. Bilirubin added to whole blood produced a corresponding linear increase in the ZPP/H ratio. However, this exogenous bilirubin effect was materially diminished by the presence of the surfactant Triton X-100, at least up to a bilirubin concentration of 70 μmol/L, which is severalfold above the health-related reference interval and would apply to most specimens. Additives that did not have similar effects on ZPP/H included albumin and the hemolyzing agents deoxycholate and saponin.

Table 1. Effects of bilirubin concentration, Triton X-100, and ProtoFluor reagent on ZPP/H.

	ZPP/H, μmol/mol		
Bilirubin added, μmol/L	Whole blood, no additives	Whole blood + Triton X-100	Whole blood + Triton X-100 and ProtoFluor reagent
0	46	39	42
17	58	31	39
35	70	32	42
70	95	39	51
87	101	44	51
99	112	52	59
174	150	75	83

A Triton X-100 concentration of 10 g/L was less effective than 40 g/L for diminishing the bilirubin interference.

Oxygenation has been a concern with hematofluorometry because oxygen-depleted hemoglobin has a shift in the absorption spectrum that leads to falsely low ZPP/H values. The ProtoFluor reagent was developed to address this problem *(12)* as well as to allow the use of aged blood specimens *(13)*. In our experience, the use of the ProtoFluor reagent gave slightly higher results, on average ~15%, in either the presence or absence of Triton X-100. On the other hand, if fresh blood is used or if erythrocytes are washed routinely, oxygenation may become irrelevant. ZPP binds to hemoglobin *(14)* and is stable for at least 1 week when blood is stored refrigerated at 20 °C. Given the hemolysis results described above, blood could presumably be stored frozen for extended periods and still give valid ZPP/H results.

Other investigators have recommended that erythrocytes be washed routinely to eliminate the plasma interference and to avoid false-positive results *(15)*. Although this has merit, washing the cells does add to the time required to perform the procedure. In our experience, clinical diagnostic work demands that only when the whole blood has a ZPP/H value above the reference range do the erythrocytes need to be washed to ensure that plasma interference does not yield a false-positive result. ZPP/H ratios within the reference range are acceptable even if some plasma interference is present because these values have no known clinical utility. Of course, routine washing may be required for research purposes, especially for research involving statistical analyses.

Many investigators consider hematofluorometry a poor diagnostic test because it often does not correlate well with other iron-status indicators (hematocrit, hemoglobin, ferritin, and transferrin saturation) and because of the analytical limitations cited above. Low correlation with other iron-status indicators, especially hemoglobin and hematocrit, usually occurs in states of marginal iron deficiency, not in iron deficiency anemia (decreased hemoglobin and hematocrit); however, the correlation also decreases with inflammation or anemia of chronic disease *(16)*, in which ferritin is increased and does not accurately reflect iron storage status. This presumed inconsistency is

clarified by recognizing that ZPP indicates the iron being delivered in the bone marrow for heme (hemoglobin) synthesis *(1)*. In other words, ZPP/H must be recognized as different from other indicators of iron status, such as stores and transport. When one considers exactly what the ratio is measuring, i.e., low marrow iron, the test becomes very specific. Thus, every abnormal ZPP/H finding is of medical importance, whether it be a result of nutritional iron deficiency, infection, blood loss, anemia of chronic disease, or accelerated erythropoiesis, to name a few underlying causes of relative iron deficiency anemia *(2)*.

Given our results in this evaluation, we believe that whole blood specimens are suitable for ZPP/H determination and that washing erythrocytes is required only on a limited basis for obtaining clinically valuable results by hematofluorometry. Furthermore, use of a detergent to ensure complete hemolysis is a valid modification of the procedure to obtain consistent results in the determination of the ZPP/H ratio. The hemolyzing agent (Triton X-100) can be combined with the ProtoFluor reagent if desired. Obviously, these hemolysis findings raise questions about the principle of front-surface fluorescence/absorbance as described for hematofluorometry *(3, 8)*. Perhaps the principle is more closely related to the right-angle fluorometry method used to study erythrocyte fluorescence *(11)*.

This work was supported in part by National Institutes of Health grant no. DK35816.

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Evaluation of Vacutainer Plus Low Lead Tubes for Blood Lead and Erythrocyte Protoporphyrin Testing,

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Lead poisoning is an important preventable environmental health problem. Blood lead concentrations as low as 100 μg/L (0.48 μmol/L) in whole blood have been shown to affect children's neuropsychologic or cognitive performance adversely *(1–3)*. In view of this, in 1991 the Centers for Disease Control and Prevention (CDC) lowered the concentration of lead in blood considered safe from 250 to 100 μg/L (from 1.21 to 0.48 μmol/L) *(4)*. This lowered threshold has been reaffirmed by the CDC in their 1997 document on screening children for lead exposure *(5)*. Before 1991, erythrocyte protoporphyrin (EP) was the standard test for screening children for lead poisoning; however, it was subsequently found to be an insensitive predictor of blood lead concentrations above the lower threshold *(6)*. However, serial EP measurements, paired with matching blood lead measurements, are still useful in managing children with lead poisoning. In the absence of additional exposure of the child to lead, the EP concentration will continue to decrease at a faster rate than is reflected by the blood lead concentration as lead reequilibrates among blood, soft tissue, and bone stores.

Another recommendation in the 1991 CDC statement was to screen all children 6 months to 6 years of age for lead poisoning, using a direct blood lead measurement. Venous blood is preferred for blood lead measurement because capillary measurements may be falsely increased by skin contamination with lead. The issue of lead contamination of capillary blood specimens obtained by fingerstick was reported earlier *(7)*. For diagnostic purposes, EDTA-containing evacuated tubes are traditionally used in collecting blood for lead and/or EP determinations. Each manufactured lot of tubes should be certified as free of analytically significant lead contamination by the blood lead testing laboratory before use. Such a practice is proposed in a recent NCCLS document on blood lead testing *(8)*. A significant lead contamination is defined as one that would increase the blood lead concentration by >5 μg/L. Alternatively, the testing laboratory may recommend the use of trace element tubes