

The Helena ProtoFluor Reagent System is designed for use with a hematofluorometer to measure zinc protoporphyrin in whole blood.

#### SUMMARY

The measurement of zinc protoporphyrin (ZPP) in the red blood cells is used as a diagnostic test for the microcytic hypochromic anemia of iron deficiency.<sup>1,4</sup> Heme is formed in the developing red cell by insertion of iron into a formed porphyrin ring. In the event of insufficient iron supply (iron deficiency) or impaired iron utilization (extreme lead intoxication), zinc is substituted for iron into protoporphyrin IX. The ZPP formed in the chelation process is stable and remains in the red cell for its 120 day life span. The level of ZPP in the red cell, then, is a functional indicator of the available iron supply at the time of cell maturation.

The presence of elevated blood lead levels also results in increased ZPP. The CDC has lowered the recommended lead screening level in children from 25 ug/dL to 10 ug/dL. Lead levels below 25 ug/dL do not significantly affect ZPP formation, and therefore the use of this test as a lead screen is no longer recommended.

Lamola and Yamane showed that the fluorescent erythrocyte porphyrin associated with extreme lead intoxication is zinc protoporphyrin (ZPP) and determined its absorption and fluorescence properties within the red blood cell.<sup>5</sup> ZPP can be detected easily in whole blood by use of a front face fluorometer.<sup>6,7</sup> The absorption (424 nm Soret band) and fluorescent maxima (595 nm) of ZPP in blood differ from those metal-free porphyrins associated with various porphyrias. The fluorescent porphyrin associated with iron deficiency anemia is ZPP which is identical to that produced by lead intoxication. During the hematofluorometer measurement of ZPP, incomplete oxygenation of the blood causes a spectral shift in the hemoglobin that leads to lower results, i.e., false negatives or low sensitivity. With the use of ProtoFluor Reagent, a stable hemoglobin spectrum is produced, avoiding the deoxygenation shift, with results comparable to full oxygenation of the hemoglobin. Without the use of ProtoFluor Reagent a gentle stream of air must be blown over the drop of blood on the test slide just prior to making the measurement. Standardization of this method is extremely difficult, and significant variations in test results are seen. ProtoFluor Calibrators are reproducible, assayed solutions designed to provide instrument calibration at high and low levels of the ProtoFluor system. They may also be used for day-to-day calibration, reproducibility and linearity for other hematofluorometer systems according to the manufacturer's specifications.

Blood porphyrin assays other than hematofluorometry involve extraction of the porphyrin(s) followed by some type of purification step.<sup>8</sup> Hematofluorometry using the ProtoFluor Reagent and ProtoFluor Calibrators provides a very simple, easy, accurate and inexpensive method for determining erythrocyte ZPP levels in the diagnosis of iron deficiency. The method requires only one drop of whole blood and no sample measurements are necessary. In addition to general laboratory diagnosis and nutritional monitoring, the test can be beneficial in specialties such as blood banking, sports medicine and pediatrics where iron status is a particular concern. The use of ZPP testing for occupational lead exposure testing has not been validated on the ProtoFluor Reagent system.

#### PRINCIPLE

The principle of hematofluorometry for measuring zinc protoporphyrin requires that the hemoglobin be fully oxygenated. By adding ProtoFluor Reagent to whole blood, the hemoglobin is derivatized to a product having the spectral characteristics of oxyhemoglobin in the region where the hematofluorometer operates. Thus, the need for oxygenation is circumvented, which allows the determination of zinc protoporphyrin with greater accuracy and precision.

#### REAGENTS

##### 1. ProtoFluor Reagent

**Ingredients:** The reagent contains a cyanide salt with stabilizers in aqueous solution.

**DANGER: POISON - NEVER PIPETTE BY MOUTH. VAPOR HARMFUL.**

Cannot be made nonpoisonous. May be fatal or cause blindness if swallowed. Call physician immediately.

**Preparation for Use:** The reagent is ready for use as packaged.

**Storage and Stability:** The reagent should be stored at 15 to 30°C and is stable until the expiration date indicated on the vial.

**Signs of Deterioration:** The reagent should be a clear colorless solution. Cloudiness maybe indicative of product deterioration.

##### 2. ProtoFluor Low Calibrator and ProtoFluor High Calibrator

**Ingredients:** The calibrators contain zinc protoporphyrin solution and pyridine with preservatives and stabilizers. The calibrators are assayed in µmol ZPP/mole Heme for the ProtoFluor system. Refer to the assay card packaged with the calibrators for the ZPP concentration.

**WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST. NEVER PIPETTE BY MOUTH**

**Preparation for Use:** The calibrators are ready for use as packaged.

**Storage and Stability:** The calibrators should be stored at 2 to 8°C and are stable until the expiration date indicated on the vials.

**Signs of Deterioration:** The calibrators should be opaque with no particulate matter present.

#### INSTRUMENTS

The Helena ProtoFluor Z hematofluorometer is designed to measure the zinc protoporphyrin/heme ratio in whole blood.

#### SPECIMEN COLLECTION AND HANDLING

**Specimen:** Whole blood collected with an anticoagulant such as heparin, EDTA, or citrate is the specimen of choice.

**Blood Collected from Fingertick:** Collect blood into heparinized micro-hematocrit capillary tubes. Seal the end of the tubes with Critoseal. When ready to perform the test, cut off the end of the tubes and expel a drop of blood into a 12 x 75 mm test tube. Proceed as directed for venipuncture samples in the STEP-BY-STEP METHOD.

**Patient Preparation:** No special patient preparation is required.

#### Interfering Substances:

1. Do not use hemolyzed or frozen specimens
2. Abnormally elevated bilirubin will create positive interference due to its spectral qualities.
3. Routine plasma washing of samples to remove interferences is not recommended.

**Storage and Stability:** Specimens should be analyzed soon after collection and before hemolysis occurs. Storage at 4°C for up to one week may be acceptable, but as anticoagulated blood ages, it becomes hemolyzed, which may cause erroneous results.

#### PROCEDURE

##### Materials Provided:

ProtoFluor Reagent System Kit-Cat. No. 2000

Contains

- ProtoFluor Reagent ( 2 x 15 mL)
- ProtoFluor Low Calibrator (1 x 2.5 mL)
- ProtoFluor High Calibrator (1 x 2.5 mL)
- ProtoFluor Coverslips (2 x 125)

##### Materials available but not contained in the kit:

Item	Cat. No.
ProtoFluor Z Hematofluorometer	2005
ProtoFluor Coverslips (10 x 125)	2001
ProtoFluor Reagent-2 (2 x 15 mL)	2002
ProtoFluor Calibrator-Low (5 x 2.5 mL)	2010
ProtoFluor Calibrator-High (5 x 2.5 mL)	2011

##### Materials Needed but not Provided:

- Glass test tubes, 12 x 75 mm
- Pasteur pipettes (glass)

#### STEP-BY-STEP-METHOD

**NOTE:** Refer to the Hematofluorometer Operator's Manual for complete instructions for measurement of ZPP levels in whole blood. The following procedure specifically addresses use of the ProtoFluor Calibrators and ProtoFluor Reagent.

- A. Allow the hematofluorometer to warm up as directed.
- B. Allow the ProtoFluor Calibrators and stored samples to equilibrate to room temperature. Fluorescence is affected by temperature; therefore, equilibration to room temperature is very important.
- C. Perform Instrument Calibration and Quality Control on the hematofluorometer using the ProtoFluor Calibrators.
  1. Calibrate the ProtoFluor Z Hematofluorometer using ProtoFluor High and Low Calibrators according to the instructions in the Operator's Reference Manual.
  2. Verify the precision of other hematofluorometer systems according to the following steps.
    - a. Place a drop of ProtoFluor Low Calibrator directly on a sample cover-slip. Using the tip of the vial, spread the drop so that the calibrator completely covers the sample area shown. DO NOT ADD ProtoFluor Reagent to the calibrator.
    - b. Make a reading in the hematofluorometer and record the results.
    - c. Repeat Steps a. and b. using ProtoFluor High Calibrator

**NOTE:** Make only one reading from each prepared slide. If a repeat reading is necessary, place a fresh drop of calibrator on a clean coverslip and make a second reading. Refer to the "Stability of End Product" Section.

- D. Determine ZPP level in whole blood samples using ProtoFluor Reagent.
- Using a Pasteur pipette, place one drop of the patient whole blood sample in a small test tube (12 x 75 mm). See SPECIMEN COLLECTION AND HANDLING for the recommended procedure for fingerstick samples.
  - Add two drops of ProtoFluor Reagent.
  - Mix by shaking briefly (a few seconds is adequate). Do not vortex.
  - Pour a drop of specimen onto a glass coverslip which has been placed into the sample holder. Spread the drop using the lip of the test tube so that the specimen covers the appropriate area of the coverslip.
  - Proceed with the measurement process as instructed by the Operator's Manual for your hematofluorometer.

#### Stability of End Product

Once the blood and reagent have been mixed, the solution is stable for up to five minutes. After a reading has been taken, the test mixture is no longer stable. Irradiation of the blood sample during reading may cause photodecomposition, making subsequent measurements from the same sample unreliable. Do not take more than one reading from a prepared slide.

#### Calibration

ProtoFluor Calibrators are provided in two levels in each ProtoFluor Reagent System Kit. These may be used for calibration or as a means of daily tracking, depending on the manufacturer's calibration procedure.

**NOTE:** Many existing hematofluorometers have no external calibration adjustments; in this case, use the calibrators as daily checks for linearity or precision.

#### Quality Control

The ProtoFluor Calibrators may serve as means for calibration or quality control. Two levels (low and high) of assayed reference materials are included with each kit.

#### EXPECTED VALUES

Use of the ProtoFluor Reagent should give results comparable to those obtained with fully oxygenated, fresh, whole blood. Actual values will depend on the units displayed by any particular instrument (i.e.  $\mu\text{g ZPP/dL}$  whole blood;  $\mu\text{g ZPP/g Hgb}$ ,  $\mu\text{mol ZPP/mol heme}$ ;  $\mu\text{g ZPP/dL RBC}$ ).

#### LIMITATIONS

See also SPECIMEN COLLECTION AND HANDLING, Interfering Substances.

- Results may be compromised if annual recommended preventative maintenance has not been performed on the hematofluorometer. Preventative maintenance must occur at Helena Laboratories. A decrease in results may occur prior to detection by calibration and optical checks.
- Samples **must be run with reagent**. Results with fresh samples may be significantly decreased when run without reagent. Moderately aged samples run without reagent may generate unpredictable results.

#### INTERPRETATION OF RESULTS

Elevated levels of ZPP in red blood cells are indicative of iron deficiency or severe lead intoxication. Iron deficiency may result from excessive blood loss or insufficient iron intake, absorption or utilization. It often occurs during periods of increased need such as during the rapid growth periods of infancy and childhood or during pregnancy. Elevated levels of ZPP may also be seen in anemia associated with chronic disease such as chronic infection or malignancy in which iron release from the reticuloendothelial cell is blocked.<sup>1</sup> In cases of iron deficiency, ZPP measurements are a means of diagnosing subclinical changes in the biosynthetic pathway of heme before anemia develops. In summary, ZPP increases with relative iron deficient erythropoiesis.

An elevated ZPP can also result from levels of lead higher than 35  $\mu\text{g/dL}$ . This observation must be considered when interpreting elevated ZPP results. Lead at this level can be extremely toxic. Behavioral problems and low IQ have been documented at lead levels too low to detect by ZPP analysis, and therefore, this test is no longer recommended for lead screening. The use of ZPP testing for occupational lead exposure testing has not been validated on the ProtoFluor system.

#### PERFORMANCE CHARACTERISTICS

##### Precision Studies

##### Run-to-Run:

	ProtoFluor Calibrators		ProtoFluor Reagent
	Low	High	
$\bar{X}$	82 $\mu\text{mol/mol}$	170 $\mu\text{mol/mol}$	104 $\mu\text{mol/mol}$
SD	6.34	3.93	10.10
CV%	2.3	2.3	9.7
n	150	207	50

**Within Run:** A single blood sample was run in replicate with the ProtoFluor Reagent with the following results.

$\bar{X}$	=	170 $\mu\text{mol/mol}$
SD	=	5.3
CV	=	4.9%
n	=	20

#### Comparison Studies

Correlation studies were done using the ProtoFluor System and Aeration. Data were generated on a custom modified hematofluorometer designed to display results as the metabolite ratio  $\mu\text{mol ZPP/mol Heme}$ . The results were as follows:

$r$	=	0.97
slope	=	0.87
y intercept	=	10.67

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