Intended Use
For use in the manual, quantitative, colorimetric determination of hemoglobin in plasma.

Clinical Significance [1, 2]
Measurement of Plasma Hemoglobin is of value in monitoring hemolytic transfusion reaction and certain other conditions associated with intravascular hemolysis. An increase in Plasma Hemoglobin is indicative of acute destruction of erythrocytes (hemolysis) within the vascular system, intravascular destruction (hemolysis) of red blood cells. It is used to evaluate hemolytic anemia, especially intravascular hemolysis. Plasma Hemoglobin is increased with intravascular hemolysis, ABO incompatible transfusion, traumatic hemolysis, falciparum malaria, burns, and march hemoglobinuria. Increase may occur in some cases of extravascular hemolysis, delayed transfusion reaction, slight increase in sickle cell anemia, and β-thalassemia.

Method Principle [3, 4]
Catachem's colorimetric procedure for the determination of free hemoglobin in plasma is based upon the peroxidase activity of hemoglobin. In this procedure, hemoglobin activates the oxidation of 3.3', 5.5'-tetramethylbenzidine by hydrogen peroxide to form a chromogenic product with maximum absorption at 650nm. The increase of absorbance is directly proportional to the concentration of hemoglobin in the plasma sample. This procedure is simple and accurate and it is based on the work of Lijana, R.C., and Williams, M.C., and Standefer, J.C. and Vanderjagt, D.

REAGENTS
Substrate Reagent
Each liter contains approximately:

Buffer	
3,3',5,5'-tetramethylbenzidine(TMB)	2.3 mmoles
Solubilizer	

Activator Reagent
Each liter contains approximately:

Hydrogen Peroxide	3 mmoles
Stabilizer	

Precautions
Handle these reagents using good laboratory practice. **DO NOT PIPETTE REAGENT BY MOUTH.** Avoid contact with skin and eyes. If contact occurs, wash affected area with plenty of cold water. Clean spills immediately. Dispose of in accordance with local regulations and laws.

TMB is harmful by inhalation in contact with skin and if swallowed. It is irritating to the eyes. Do not breathe vapor. If contact occurs with eyes, rinse immediately with plenty of cold water. Seek medical attention. TMB is a possible mutagen. Refer to the MSDS for additional information.

Reagent Storage and Stability
Store the unopened Catachem Plasma Free Hemoglobin reagents at 2-8°C. When stored as directed, the reagents are stable until expiration date stated on the label.
Once opened, the reagents are stable for at least 60 days stored at 2-8ºC and capped while not in use.

Reagent Preparation
Catachem Plasma Free Hemoglobin reagents are packaged in ready-to-use form. No preparation is required.

Reagent Indications of Deterioration
- Turbidity
- Absorbance > 0.5 OD, 1 cm light path, 650nm
- Quality control values out of assigned ranges

If these reagent characteristics are observed, contact Catachem technical service.

Specimen Collection and Stability [6]
Clear unhemolyzed plasma collected in heparin as anticoagulant is the specimen of choice. Blood should be drawn without trauma and handled carefully to avoid hemolysis. Hemolysis may occur if blood is drawn too quickly into the collection tube or subjected to vigorous mixing. Testing should be done promptly after specimen collection. If testing is delayed, store plasma specimen, removed from the blood cells, frozen at -20ºC. Specimens collected with anticoagulants such as EDTA, Oxalate, and others should not be used since they are known to interfere with the hemoglobin-peroxide reaction.

Procedure
These instructions are outlined for performing the Catachem Plasma Free Hemoglobin assay manually. Read the entire procedure instructions before performing this assay.

Materials Provided
Catachem Plasma Free Hemoglobin Substrate Reagent
Catachem Plasma Free Hemoglobin Activator Reagent

Materials Required But Not Provided
- Spectrophotometer equipped with 650nm wavelength
- Calibrator material with assigned PFH values
- Calibrator and quality control material with assigned Plasma Free Hemoglobin values

Calibration
Catachem Plasma Free Hemoglobin Calibrator, Product No. C464-20, which contains a known Plasma Hemoglobin value, is recommended.

Calibration Schedule
Calibration should be performed when this method is implemented for the first time and every time the procedure is repeated thereafter.

Quality Control
To monitor the quality performance of the procedure, Catachem recommends the use of Catachem Plasma Free Hemoglobin Control Level I and Control Level II, Product Nos. C464-21 and C464-22, with assigned Plasma Free Hemoglobin values. These

quality control materials should be included in the assay each time the procedure is performed.

Analytical Parameters

Wavelength	650nm
Temperature	37˚C
Path length	1 cm
Reaction Mode	End Point Rate
Reaction Time	3 minutes
Reaction Volume (R1)	0.5 mL
Reaction Volume (R2)	0.2 mL
Sample Volume	0.006 mL
Total Volume	0.706 mL
Sample-to-reagent ratio	1:118

Assay Procedure

1. Prepare the Plasma Free Hemoglobin Reagents by following instructions under "Reagent Preparation".
2. Set spectrophotometer temperature at 37ºC, wavelength at 650nm and zero the instrument with a reference cuvette containing water.
3. Pipette 0.5 mL of Substrate Reagent into each of four cuvettes marked: "Blk", "Test", "Calibrator" and "Control".
4. Pipette 0.006 mL of Blk (water), Test sample, Calibrator and Control into their respective cuvettes.
5. At timed intervals, add 0.20 mL of Activator Reagent to each reaction cuvette. Mix each cuvette immediately by inversion.
6. Exactly **1 minute** after addition of Activator Reagent read absorbance (A) of each cuvette marked BLK, Test, Calibrator and Control at the set wavelength of 650nm against water as reference.

Catachem Plasma Free Hemoglobin Procedure Scheme				
	RGT BLK	**CAL**	**CONTROL**	**TEST**
Substrate	**0.5 mL**	**0.5 mL**	**0.5 mL**	**0.5 mL**
Water	**0.006 mL**	**-**	**-**	**-**
Sample	**-**	**0.006mL**	**0.006mL**	**0.006mL**
Activator	**0.2 mL**	**0.2 mL**	**0.2 mL**	**0.2 mL**
Mix	**All cuvettes**			
Incubate	**All cuvettes at 37º C**			
Read OD	**Exactly one minute after addition of Activator**			
Calculate	**Hemoglobin in mg/dL**			

Calculations and Results:

$$\text{UN (mg/dL)} = \frac{\text{(A) Sample - (A) Blank}}{\text{(A) Calibrator- (A) Blank}} \times \text{calibrator (mg/dL)}$$

Example:

(A) Blank	(A) Test	(A) Calibrator
0.05	0.25	0.50

Calibrator = 25 mg/dL

$$\text{Unknown PFH test value} = \frac{0.25 - 0.05}{0.50 - 0.05} \times 25 = 11.1 \text{ mg/dL}$$

Procedure Limitations

Samples with Plasma Hemoglobin values greater than 100mg/dL should be diluted with physiological saline and re-assayed. Multiply results obtained by dilution factor.

Interfering Substances [7]

The following substances have no significant effect on the accuracy of this Plasma Hemoglobin procedure at the concentrations stated.

- Bilirubin ≤ 20mg/dL
- Ascorbic Acid ≤ 2mg/dL

Other factors such as hemolysis during and after venipuncture, lipemic plasma, turbidity, and methemalbuminemia may cause falsely elevated values in the Plasma Hemoglobin assay.

Expected Values [5]

Plasma Hemoglobin healthy subjects ≤ 5mg/dL

The values given here are only to be used as a guideline. It is recommended that each laboratory establish the normal range for the geographical area in which it is located.

Method Performance Characteristics

Sensitivity: Using a path length of 1 cm, a Δ-absorbance of 0.03 - 0.028 per g/dL should be obtained.

Linearity: This procedure is linear over the range of 0 - 100mg/dL.

Precision:

PFH	Within-Run		Day-Day	
Mean	**SD**	**CV**	**Mean**	**SD**
mg/dL	**mg/dL**	**%**	**mg/dL**	**%**
3.9	**0.15**	**3.98**	**0.17**	**4.92**
26.4	**0.90**	**3.42**	**1.16**	**4.51**
54.40	**1.53**	**2.81**	**1.19**	**3.71**

Bibliography

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