



TOTAL HEMOGLOBIN (Procedure No. 525)

INTENDED USE

Sigma Diagnostics Total Hemoglobin reagents are intended for the quantitative, colorimetric determination of hemoglobin concentration in whole blood at 530–550 nm.

BACKGROUND AND PRINCIPLE OF TEST

Classic techniques for determining blood hemoglobin were based on estimation of oxygen, carbon monoxide capacity or iron content. These assays proved unreliable because of the heterogeneous nature of hemoglobin. Stadie¹ proposed a colorimetric cyanmethemoglobin method where total hemoglobin at alkaline pH is rapidly converted to the cyanoderivative. The absorbance of the cyanoderivative is determined at 540 nm. Drabkin and Austin² simplified the method by combining the separate reactants, alkaline ferricyanide and cyanide, into a single reagent.

Results from field trial of a cyanmethemoglobin standard by the U.S. Army^{3,4} prompted the National Institute of Health to recommend the cyanmethemoglobin method to the National Research Council (NRC) for further investigation. In 1958, the NRC advocated adoption of the cyanmethemoglobin procedure for blood hemoglobin in the U.S. and Canada. In 1966 the International Committee on Standardization in Hematology approved the proposal that clinical laboratories should adopt the cyanmethemoglobin method exclusively for quantitation of hemoglobin in blood.⁵

Drabkin's solution reacts with all forms of hemoglobin, except sulfhemoglobin, a pigment that normally occurs only in minute concentration in blood. The broad absorption peak of cyanmethemoglobin permits its measurement using both wide and narrow bandwidth instruments.

Initially the cyanmethemoglobin solutions were considered to be the most stable form of hemoglobin standard; but after a decade of experience, it was shown that dilute solutions of cyanmethemoglobin may be unstable.^{6,7} Riggs and Wolbach⁸ established that alkaline solutions of hemoglobin deteriorate due to oxidation of sulfhydryl groups. The hemoglobin standard was found to be stable if stored in the form of lyophilized methemoglobin.⁹ When the lyophilized hemoglobin standard is reconstituted with Drabkin's solution, it provides a reliable, stable cyanmethemoglobin standard solution.

Sigma provides a stable, dry Drabkin's reagent containing a surfactant to minimize turbidity sometimes caused by hyperproteinemia, hyperlipemia or the presence of erythrocyte stroma. The Sigma procedure is based on the oxidation of hemoglobin and its derivatives (except sulfhemoglobin) to methemoglobin in the presence of alkaline potassium ferricyanide. Methemoglobin reacts with potassium cyanide to form cyanmethemoglobin which has maximum absorption at 540 nm. The color intensity measured at 540 nm is proportional to the total hemoglobin concentration.

REAGENTS

DRABKIN'S REAGENT, Catalog No. 525-2
Sodium bicarbonate, 100 parts, potassium ferricyanide, 20 parts, and potassium cyanide, 5 parts.

BRJ-35 SOLUTION, Catalog No. 430AG-6
Brj-35, 30 g/dL. If solution solidifies, warm to 37°C to liquefy.

HEMOGLOBIN STANDARD, Catalog No. 525-18
Lyophilized human methemoglobin. Equivalent to hemoglobin concentration of 18 g/dL whole blood when reconstituted and used according to this procedure.

PRECAUTIONS:

Sigma Diagnostics Hemoglobin reagents are for "In Vitro Diagnostic Use". Normal precautions exercised in handling laboratory reagents should be followed. Dispose of waste observing all local, state and federal laws.

Drabkin's Reagent and Drabkin's Solution are HIGHLY TOXIC. Very toxic by inhalation in contact with skin and if swallowed. Risk of serious damage to eyes. Irritating to skin. Contact with acids liberates very toxic gas. If you feel unwell, seek medical advice (show the label where possible). Wear suitable protective clothing, gloves and eye/face protection. Do not breathe dust. Target organs: blood and central nervous system.

Hemoglobin Standard is a POTENTIALLY BIOHAZARDOUS MATERIAL. Source material from which this product was derived was found negative for hepatitis B surface antigen and for the antibody to HIV by required test methods. However, since no test method can offer complete assurance that hepatitis B virus, HIV or other infectious agents are absent, this product should be handled observing the same safety precautions employed when handling any potentially infectious material.

Refer to Material Safety Data Sheet for any updated risk, hazard or safety information.

PREPARATION:

DRABKIN'S SOLUTION is prepared by reconstituting Drabkin's Reagent, Catalog No. 525-2, with 1000 mL deionized water. Add 0.5 mL 30% Brj-35 Solution, Catalog No. 430AG-6, and mix well. Filter if insoluble particles remain.

CYANMETHEMOGLOBIN STANDARD SOLUTION is prepared by reconstituting vial of Hemoglobin Standard, Catalog No. 525-18, with 50.0 mL Drabkin's Solution. Mix well and allow to stand for at least 30 minutes.

STORAGE AND STABILITY:

Store Drabkin's Reagent in the dark at room temperature (18–26°C). Stable until the expiration date shown on the label.

Store Brj-35 Solution at room temperature (18–26°C).

Store Hemoglobin Standard in refrigerator (2–8°C). Stable until the expiration date shown on the label.

Drabkin's Solution is stable for at least 6 months stored in amber bottle at room temperature (18–26°C).

Cyanmethemoglobin Standard Solution is stable for at least 6 months stored in refrigerator (2–8°C).

DETERIORATION:

Discard Brj-35 Solution if turbidity develops.

SPECIMEN COLLECTION AND STORAGE

It is recommended that specimen collection be carried out in accordance with NCCLS document M29-T2. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, all blood derivatives should be considered potentially infectious.

Capillary or venous blood may be collected. Specimens obtained by capillary puncture should be free flowing and pipetted immediately into Drabkin's Solution, rinsing the pipet 3–4 times in the reagent. Venipuncture specimens must be collected in tubes containing solid anticoagulants, such as oxalate, citrate, EDTA or heparin. After thorough mixing with the anticoagulant, blood can be frozen for as long as 2 years¹⁰ or stored for at least a week at 30°C.¹¹

INTERFERING SUBSTANCES:

Substances that cause turbidity will influence absorbance measurement of the cyanmethemoglobin. These include lipids,¹¹ abnormal plasma proteins¹² or erythrocyte stroma.¹³

MANUAL PROCEDURE

MATERIAL PROVIDED: See "Reagents" section.

MATERIALS REQUIRED BUT NOT PROVIDED:

Instrument: Any filter photometer or spectrophotometer that transmits light at 540 nm is suitable.

Materials: Test Tubes, 10 mL

Pipets, 20 µL Sahli type or micro; 10 mL serologic

PROCEDURE:

1. Label two or more test tubes, BLANK, TEST 1, TEST 2, etc.
2. To all tubes, add 5.0 mL Drabkin's Solution.
3. To TEST, add 20 µL whole blood, rinsing pipet 3–4 times with reagent. Mix well. Allow to stand at least 15 minutes at room temperature (18–26°C). NOTE: Specimens with appreciable carboxyhemoglobin content such as those obtained from smokers may require a longer reaction time under the conditions of the test. Alternatively, it has been suggested that in those cases, warming the reaction mixture at 56°C for 3–5 minutes with gentle mixing will bring the reaction to completion.¹⁴
4. Read and record absorbance (A) of TEST vs BLANK as reference at the same wavelength and in the same instrument as used in the preparation of your calibration curve.
5. Determine total hemoglobin concentration (g/dL) of TEST directly from your calibration curve. Color is stable for several hours.

CALIBRATION:

The Hemoglobin Standard, Catalog No. 525-18, is offered as a dry vial containing a standardized amount of methemoglobin prepared from human hemoglobin. In standardizing the product, the millimolar absorptivity of cyanmethemoglobin at 540 nm and the molecular weight of hemoglobin are taken to be 44.0 and 64,458, respectively.⁵ Reconstituting the Hemoglobin Standard, Catalog No. 525-18, as directed yields the Cyanmethemoglobin Standard Solution. This solution will yield an absorbance equivalent to that of a whole blood sample containing a hemoglobin level of 18 g/dL that has been diluted 1:251 with Drabkin's Solution. Dilutions of the Cyanmethemoglobin Standard Solution with Drabkin's Solution are used to prepare a calibration curve as follows:

1. Prepare working standards by pipetting and mixing thoroughly the solutions indicated below.

1	2	3	4
Tube No.	Cyanmethemoglobin Standard Solution (mL)	Drabkin's Solution (mL)	Blood Hemoglobin (g/dL)
1	0.0	6.0	0.0
2	2.0	4.0	6.0
3	4.0	2.0	12.0
4	6.0	0.0	18.0

NOTE: Diluted standards are stable for as long as 6 months when stored tightly capped and refrigerated (2–8°C) in the dark.

$$Y = 0.0025X + 0.005$$

2. Read absorbance of Tubes 2-4 vs Tube 1 as reference at a wavelength between 530-550 nm.
3. Record absorbance values.
4. Plot a calibration curve of absorbance values vs blood hemoglobin (g/dL) in Column 4. The curve is linear, passing through the origin.

QUALITY CONTROL:

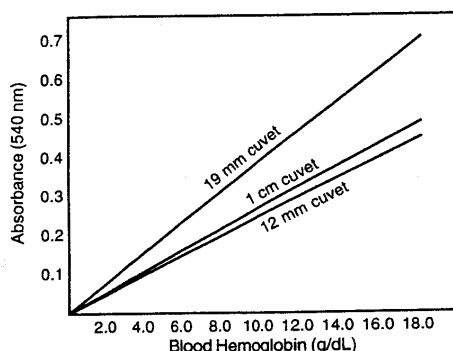
Quality control may be monitored by use of frozen aliquots of pooled whole blood.¹⁰ Commercially available blood preparations with assayed hemoglobin values are also suitable for control purposes.

RESULTS

Results are obtained directly from the calibration curve.

EXAMPLE: A specimen assayed by this procedure yielded an absorbance of 0.395 using 19-mm cuvet. From "Typical Calibration Curves" (Figure 1) a blood hemoglobin value of 10 g/dL is obtained.

Figure 1. Typical Calibration Curves



NOTE: Typical calibration curves (Figure 1) are presented for illustrative purposes and must not be used to calculate test results.

EXPECTED VALUES

NORMAL RANGE¹¹

	Blood Hemoglobin (g/dL)
Adult Males	13 - 18
Adult Females	11 - 16
Newborns	14 - 23

A number of factors, such as age, race, exercise, season and altitude, are reported to influence the values of normal ranges as shown above.¹¹ Increased hemoglobin values are observed in cases of primary and secondary polycythemia, severe dehydration, congestive heart failure and in blood from persons living at high altitudes.¹⁵ Low values are encountered in a variety of anemias. A comprehensive review by Young¹⁶ reveals that numerous drugs exert an in vivo effect to give decreased whole blood hemoglobin values.

PERFORMANCE CHARACTERISTICS

Reproducibility studies were performed on diluted and undiluted whole blood specimens with total hemoglobin concentrations of 5.3, 8.0 and 16.1 g/dL. Each specimen was analyzed on 10 separate occasions. The standard deviations and coefficients of variation were found to be 0.02, 0.08, 0.06 g/dL and 0.4, 1.0, 0.4%, respectively.

Correlation studies were conducted with 3 groups of whole blood samples using the described technique and indicated reference methods. The following hemoglobin values for these methods, as well as for the Sigma procedure were obtained.

Reference Method	Number of Specimens	Mean Hemoglobin (g/dL)		Correlation Coefficient
		Reference	Sigma	
Spectrophotometric	15	14.5	14.5	0.998
AutoAnalyzer®	52	11.3	11.2	0.976
Coulter S®	30	12.3	12.3	0.965

AutoAnalyzer is a registered trademark of Technicon Corporation
Coulter S is a registered trademark of Coulter Electronics

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REFERENCES

1. Stadie WC: A method for the determination of methemoglobin in whole blood. *J Biol Chem* 41:237, 1920
2. Drabkin DL, Austin JH: Spectrophotometric studies. II. Preparations from washed blood cells; nitric oxide hemoglobin and sulfhemoglobin. *J Biol Chem* 112:51, 1935
3. Crosby WH, Munn JL, Furth FW: Standardizing a method for hemoglobinometry. *US Armed Forces Med J* 5:693, 1954
4. Crosby WH, Houchin DN: Preparing standard solutions of cyanmethemoglobin. *Blood* 12:1132, 1957
5. Eilers RJ: Notification of final adoption of an international method and standard solution for hemoglobinometry specifications for preparation of standard solution. *Am J Clin Pathol* 47:212, 1967
6. Cannan RK: Proposal for a certified standard for use in hemoglobinometry: Second and final report. *Blood* 13:1101, 1958
7. Von Klein-Wisenberg A, Clotten R, Freiburg B: Examination of commercial haemoglobincyanide standard solutions by spectrophotometry, electrophoresis, and chromatography. *Bibl Haematol* 21:79, 1965
8. Riggs AF, Wolbach RA: Sulfhydryl groups and the structure of hemoglobin. *J Gen Physiol* 39:585, 1956
9. Data obtained by Sigma Diagnostics
10. Schoen I, Solomon M: Control of blood haemoglobin determination by a simple effective method. *J Clin Pathol* 15:44, 1962
11. Makarem A: Hemoglobins, Myoglobins, and Haptoglobins. IN *Clinical Chemistry - Principles and Technics*, 2nd ed. RJ Henry, DC Cannon, JW Winkelman, Editors, Harper & Row, Hagerstown (MD), 1974, pp 1128-1135
12. Green P, Teal CFJ: Modification of the cyanmethemoglobin reagent for analysis of hemoglobin in order to avoid precipitation of globulins. *Am J Clin Pathol* 32:216, 1959
13. Van Kampen EJ, Zijlstra WG: Standardization of hemoglobinometry. II. The hemoglobincyanide method. *Clin Chim Acta* 6:538, 1961
14. Rice EW: Rapid determination of total hemoglobin as hemoglobin cyanide in blood containing carboxyhemoglobin. *Clin Chim Acta* 18:89, 1967
15. Hepler OE: *Manual of Clinical Laboratory Methods*, 4th ed. CC Thomas, Springfield (IL), 1957, p 40
16. *Effects of Drugs on Clinical Laboratory Tests*, 3rd ed. DS Young, Editor, AACCPress, Washington (DC), 1990; Supplement No. 1, 1991

REAGENTS FOR DETERMINATION OF BLOOD HEMOGLOBIN

KIT		
Catalog No.		525-A
Maximum Assays		1000
Contents - Catalog Numbers		
Drabkin's Reagent, 525-2		6 vials
Hemoglobin Standard, 525-18		2 x 1 vial
Brij-35 Solution, 430AG-6		10 mL
INDIVIDUAL REAGENTS		
Catalog No.	Item	Quantity
525-2	DRABKIN'S REAGENT (Vial reconstitutes to 1000 mL)	6 vials
525-18	HEMOGLOBIN STANDARD	1 vial
430AG-6	BRIJ-35 SOLUTION	10 mL

Total Hemoglobin
Procedure No. 525
Previous Revision: July 1994
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