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## Reference and Selected Procedures for the Quantitative Determination of Hemoglobin in Blood; Approved Standard—Third Edition

This document describes the principle, materials, and procedure for reference and standardized hemoglobin determinations. It includes specifications for secondary hemiglobincyanide (HiCN) standards.

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A standard for global application developed through the NCCLS consensus process.



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## Reference and Selected Procedures for the Quantitative Determination of Hemoglobin in Blood; Approved Standard—Third Edition

### Abstract

NCCLS document H15-A3, *Reference and Selected Procedures for the Quantitative Determination of Hemoglobin in Blood; Approved Standard—Third Edition*, describes the measurement of blood hemoglobin using the hemiglobincyanide (HiCN) method, including composition of, and criteria for, the reagent and the calibration of photometers. The procedures described in H15 are required for whole blood calibration procedures for automated hematology analyzers; are necessary in the evaluation of instruments and alternative methods for the determination of hemoglobin concentration; and should be applied when patient red blood cell measurements are used for calibration and control of hematology analyzers. A separate section contains specifications for, and spectral characteristics of, HiCN solutions suitable for use as standards. The document enables users to achieve accurate hemoglobin concentration values for diagnostic or reference purposes. Producers of HiCN calibration standards can use the document as a guideline; users will have the information necessary to check for the content and purity of those materials.

NCCLS. *Reference and Selected Procedures for the Quantitative Determination of Hemoglobin in Blood; Approved Standard—Third Edition*. NCCLS document H15-A3 (ISBN 1-56238-425-2). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2000.

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## Foreword

“Although the determination of the amount of haemoglobin is one of the most important of all chemical tests of the blood, as a rule it is one which is determined with less care and by methods more inaccurate than those in use for any other constituent of the body.”<sup>1</sup> There have been many advances in methodology and in instrumentation since the above statement was published. The biggest step forward is undoubtedly the development and acceptance of a reference method for hemoglobin determination and the availability of calibration standards.

In 1958, a Panel on the Establishment of a Hemoglobin Standard, Division of Medical Sciences—National Research Council, reviewed several photometric methods used for determining hemoglobin levels and concluded that, for several reasons, the best method was that in which hemoglobin was measured after conversion to cyanmethemoglobin:

*The method involves dilution with a single reagent. All forms of hemoglobin likely to occur in circulatory blood, with the exception of sulfhemoglobin, are determined. The color is suitable for measurement in filter as well as in narrow-band spectrophotometers, because its absorption band at a wavelength of 540 nanometers is broad and relatively flat. Standards prepared from either crystalline hemoglobin or washed erythrocytes and stored in a brown glass container and in sterile condition are stable for at least nine months (change 2%).*

Criteria for a United States cyanmethemoglobin standard were then published.<sup>2</sup>

Work continued, primarily in Europe, on the determination of the relative molecular mass of hemoglobin and on the accurate determination of the (quarter) millimolar absorptivity of hemiglobincyanide (cyanmethemoglobin). In 1963, a Standardizing Committee of the European Society of Haematology was founded; in 1964, this committee became the International Committee for Standardization in Haematology (ICSH) and an ICSH Expert Panel on Haemoglobinometry was formed to draw up recommendations. Recommendations were accepted at the International Congress of Haematology in 1966 and published in 1967. Meanwhile, the National Institute of Public Health in the Netherlands prepared and made available, on behalf of ICSH, an international hemiglobincyanide (HiCN) reference solution, one lot of which was accepted by the World Health Organization (WHO) as the International HiCN Reference Preparation (WHO Techn. Rep. Series 384: 85, 1968). WHO subsequently accepted further batches of HiCN reference solution as the second, third, etc., International HiCN Reference Preparation (now, reference standard). The international HiCN reference solutions are controlled by laboratories in Italy, Japan, the Netherlands, the United Kingdom, and the United States.

Other methods for the determination of hemoglobin were described over the past decade:<sup>3-5</sup> The HiCN method, however, remains the benchmark against which all other methods are evaluated.

In the United States the Health Care Financing Administration, U.S. Public Health Service, Department of Health and Human Services, in February 1992, published the final rule of the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88).<sup>6</sup> In this final ruling, clinical laboratory tests are categorized as either “waived tests,” “tests of moderate complexity,” or “tests of high complexity.” Waived tests include the “screening” of blood for hemoglobin concentration below or above a certain cut-off level by means of copper sulfate testing. On the recommendation of the Clinical Laboratory Improvement Advisory Committee, determination of hemoglobin concentration using whole blood collected into disposable cuvettes that contain reagents in dried form and measuring with a simple, portable, dedicated photometer, was recently added to the list of waived tests. Tests of moderate complexity include the determination of hemoglobin as part of automated hematology procedures, with or without white cell differential counting, *that do not require operator intervention during the analytic process* and do not require an analyst to

## Foreword (Continued)

interpret a histogram or scattergram. All other methods to determine hemoglobin concentration, including the (manual) reference procedure, are considered to be tests of high complexity.

The first part of this standard contains guidelines for the accurate measurement of blood hemoglobin concentration using the hemiglobincyanide method. It includes composition of, and criteria for, the reagent and the calibration of photometers; routine filtration of HiCN solutions to fully reduce all background turbidity; and a need to demonstrate, in the reference procedure, that a particular instrument-cuvette combination does not show apparent light absorption by the reagent. The procedures described in H15 are required for whole blood calibration procedures for automated hematology analyzers; are necessary in the evaluation of instruments and alternative methods for the determination of hemoglobin concentration; and should be applied when patient red blood cell measurements are used for calibration and control of hematology analyzers. This section is intended to provide all laboratory personnel with a thorough understanding of the HiCN method and to enable them to obtain accurate hemoglobin concentration values for diagnostic and for reference purposes.

The second part of this standard contains specifications for, and the spectral characteristics of, HiCN solutions suitable for use as secondary photometer calibration material. It includes the calculation of the HiCN content from spectrophotometric measurements. This section was written as a guideline for producers of HiCN calibration materials and to allow users of such materials to check for content and purity of the HiCN solutions.

Please note the following changes in this document: in [Section 6.2](#) on Reagents, a description of interferences in the method has been included ([Section 6.2.6](#)). In Part II, a section on storage of HiCN standards ([Section 3.7](#)) and a section on source material other than human blood to prepare HiCN standards ([Section 5](#)) have been included.

## Key Words

Calibrator, hemiglobincyanide (HiCN), hemoglobin, hemoglobinometry, reference method

## Reference and Selected Procedures for the Quantitative Determination of Hemoglobin in Blood; Approved Standard—Third Edition

### Part I. Reference and Selected Procedures for the Determination of Hemoglobin Concentration

#### 1 Introduction

Recommendations for the determination of hemoglobin concentration in human blood were prepared by the International Council (previously Committee) for Standardization in Haematology (ICSH) in 1978,<sup>7</sup> 1987,<sup>8</sup> and 1996.<sup>9</sup> The photometric determination of hemiglobincyanide [HiCN; cyanmethemoglobin (see Section 5)] is recommended as the reference method:

*If any other method is used in routine measurement (for example, photometric determination of oxyhaemoglobin or haemoglobinazide; iron determination), it should be adjusted to obtain comparability with the haemiglobincyanide method. The determination of haemoglobin as haeminchloride (acid haematin) is not recommended because of the unreliability of this method.*<sup>7,8</sup>

#### 2 Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to “standard precautions.” Standard precautions are new guidelines that combine the major features of “universal precautions and body substance isolation” practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80.), (MMWR 1987;36[suppl 2S]:2S-18S) and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management of blood-borne exposure, refer to NCCLS document [M29—Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue](#).

#### 3 Scope

Part I of this standard describes the determination of hemoglobin concentration in human blood by the HiCN (cyanmethemoglobin) method. Accurate determination of hemoglobin concentration is:

- required for whole blood calibration procedures of automated hematology analyzers;
- necessary for the assignment of values to control materials used in hemoglobin measurement procedures;
- necessary in the evaluation of instruments and alternative methods for the measurement of hemoglobin concentration;

- applicable when patient red cell measurement means are used for calibration or control of hematology analyzers<sup>10,11</sup>; and
- applicable in the routine hematology laboratory for diagnostic purposes and for monitoring the progress of therapy.

Thus, this standard is intended for all clinical laboratory personnel and for manufacturers of instruments, reagents, and calibration and control materials for the measurement of hemoglobin concentration.

## 4 Definitions<sup>a</sup>

Terms used in this document adhere strictly to the following definitions:

**Absorbance (symbol *A*), *n*** - The logarithm of the ratio of radiant power (*I*<sub>o</sub>) incident on the sample to the radiant power (*I*) transmitted by the sample.<sup>12</sup>

$$A = \log \frac{I_o}{I}$$

Alternative terms sometimes used are “extinction” and “optical density.”<sup>13</sup> The wavelength at which the absorbance is measured can be shown as a superscript, the component of which the absorbance is measured as subscript, e.g.,

$$A_{\text{HiCN}}^{540} = \text{absorbance of hemoglobincyanide at 540 nm}$$

**Certified reference material (CRM), *n*** - A reference material that has one or more values certified by a technically valid procedure and is accompanied by, or is traceable to, a certificate or other document that is issued by a certifying body; **NOTE:** The term “Standard Reference Material” (SRM) is the name of a certified reference material (CRM), which is the trademark name of a certified reference material that has been certified and is distributed by the National Institute of Standards and Technology (NIST), a U.S. government agency formerly known as the National Bureau of Standards (NBS).

**Direct reading photometer, *n*** - A photometer whose measurement scale has been calibrated directly in units of hemoglobin concentration. These units may be gram per liter (g/L), or millimole per liter (mmol/L). An alternative term sometimes used is “hemoglobinometer.”

**Hemiglobin (symbol *Hi*), *n*** - Hemoglobin in which the iron atoms have been oxidized to the ferric state. Alternative terms used are “methemoglobin” and “ferrihemoglobin.”<sup>13</sup>

The subcommittee prefers to continue to use the term “hemiglobin” (compare “hemoglobins”) because it clearly indicates the oxidized state of the iron atom (compare: cupri, cupro; ferri, ferro; mercuri, mercurio) and allows for more simple symbols: HiCN versus CNmetHb; HiN<sub>3</sub> versus N<sub>3</sub>metHb.

**Hemoglobincyanide (symbol *HiCN*), *n*** - Hemoglobin in which the iron atoms have been oxidized to the ferric state and which has then been bonded with cyanide ions. Alternative terms used are “cyanmethemoglobin,” “cyanferrihemoglobin,” and “methemoglobincyanide.”<sup>7,13</sup>

**Hemoglobins, *n*** - All those hemoglobin derivatives normally present in circulating blood. They include deoxyhemoglobin (HHb), oxyhemoglobin (O<sub>2</sub>Hb), carboxyhemoglobin (COHb), and hemiglobin [Hi;

<sup>a</sup> Some of these definitions are found in NCCLS document NRSCL8—*Terminology and Definitions for Use in NCCLS Documents*. For complete definitions and detailed source information, please refer to the most current edition of that document.

methemoglobin (metHb)]. Please see the current edition of NCCLS document [C46—Blood Gas and pH Analysis and Related Measurements](#), for more detailed information.

**International reference preparation, *n*** - *International Standard*. A substance that has been characterized by chemical or physical means and that provides a measure against which national reference preparations and calibrators can be controlled. International reference materials are not intended to be used in laboratory working procedures but serve as materials against which commercial products can be verified and evaluated.<sup>14</sup>

**Lambert-Beer's law, *n*** - The absorbance of a homogeneous sample containing an absorbing substance is directly proportional to the concentration (*c*) of the absorbing substance and to the thickness (*l*) of the sample in the optical path.

$$A = \epsilon \cdot c \cdot l$$

Strictly speaking, this law is applicable to true monochromatic light only.

**Mean corpuscular hemoglobin (MCH), *n*** - The average amount of hemoglobin contained within the individual red cell; **NOTE:** The MCH, expressed as the amount of hemoglobin in picogram ( $10^{-12}$  gram), is calculated as follows:

$$MCH \text{ (pg)} = \frac{\text{Hemoglobin concentration (g / L)}}{\text{Number of Red Cells (per L)}}$$

**Mean corpuscular hemoglobin concentration (MCHC), *n*** - The average hemoglobin concentration within the red blood cells; **NOTE:** The MCHC, expressed as the amount of hemoglobin in gram per liter (or deciliter) of red cells, is calculated as follows:

$$MCHC \text{ (g / L)} = \frac{\text{Hemoglobin concentration (g / L)}}{\text{Packed Cell Volume (PCV, as fraction)}}$$

**(Milli)molar absorptivity (symbol  $\epsilon$ ), *n*** - The absorbance, at a given wavelength, of an absorbing substance in a concentration of 1 (m)mol/L, measured with a sample thickness of 1.000 cm. Terms used previously are “(milli)molar absorption coefficient” and “(milli)molar extinction coefficient.” The wavelength can be shown as a superscript and the component measured as a subscript, e.g.,

$$\epsilon_{\text{HiCN}}^{540} = \text{the (milli)molar absorptivity of hemoglobincyanide at 540 nm}$$

In the case of HiCN, the term “quarter (milli)molar absorptivity” is usually used, 1 mol of hemoglobin being defined as the monomer, consisting of one heme group plus one globin ( $\alpha$  or  $\beta$ ) chain.

**Photometer, *n*** - A device designed to furnish the ratio, or a function thereof, of the radiant power of two electromagnetic beams; these two beams may be separated in time, space, or both.<sup>12</sup> The instrument usually cannot isolate spectral bands narrower than 10 nm. Alternative terms used are “filter photometer” and “colorimeter.”

**Primary standard (calibrator), *n*** - A reference material that is of fixed and known chemical composition and capable of being prepared in essentially pure form. Alternatively, any certified reference material that is generally accepted or officially recognized as the unique standard for the assay, regardless of its level of purity of analyte content.<sup>14</sup>

**Reference method, *n*** - A clearly and exactly described technique for a particular determination which, in the opinion of a defined authority, provides sufficiently accurate and precise laboratory data for it to be used

to assess the validity of other laboratory methods for this determination. The accuracy of the reference method must be established by comparison with a definitive method where one exists, and the degree of inaccuracy and imprecision must be stated.<sup>14</sup> See “[selected method.](#)”

**Secondary standard (calibrator), *n*** - A substance or device that is based on a reference preparation or in which the analyte concentration or other quantity has been determined by an analytical procedure of stated reliability. Calibrators are used to calibrate, graduate, or adjust a measurement. An alternative term is “secondary calibration standard.”<sup>14,15</sup>

**Selected method, *n*** - A procedure, the reliability of which has been validated by a collaborative study and which is recommended by a defined authority for routine use in a laboratory analysis, having been selected on the grounds of its accuracy and precision, the intended scope of the test, economy of labor and materials, and ease of operation.<sup>14</sup>

**Spectral slitwidth, *n*** - The total wavelength range, in nanometer, emerging from the monochromator exit slit.

**Spectrophotometer, *n*** - An instrument with an entrance slit and one or more exit slits, with associated equipment, designed to furnish the ratio, or a function thereof, of the radiant power of two beams as a function of spectral position. The two beams may be separated in time, space, or both.<sup>12</sup> The instrument is capable of isolating spectral bands narrower than 10 nm.

**Transmittance (symbol *T*), *n*** - The ratio of radiant power (*I*) transmitted by a sample to the radiant power (*I<sub>o</sub>*) incident on the sample.<sup>12</sup>

$$T = \frac{I}{I_o}$$

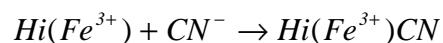
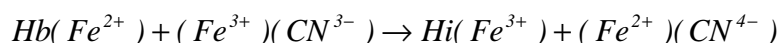
Transmittance is usually expressed as percent transmittance, *T* (%). An alternative term is “transmission.”

$$T (\%) = \frac{I}{I_o} \cdot 100$$

**Turbidity, *n*** - The condition that exists when a liquid sample contains insoluble matter of sufficient particle size to cause part of the incident light upon the sample to be scattered;<sup>16</sup> **NOTE:** Turbidity causes erroneously high absorption measurements; the magnitude of the error depends on the optical geometry of the measuring instrument.

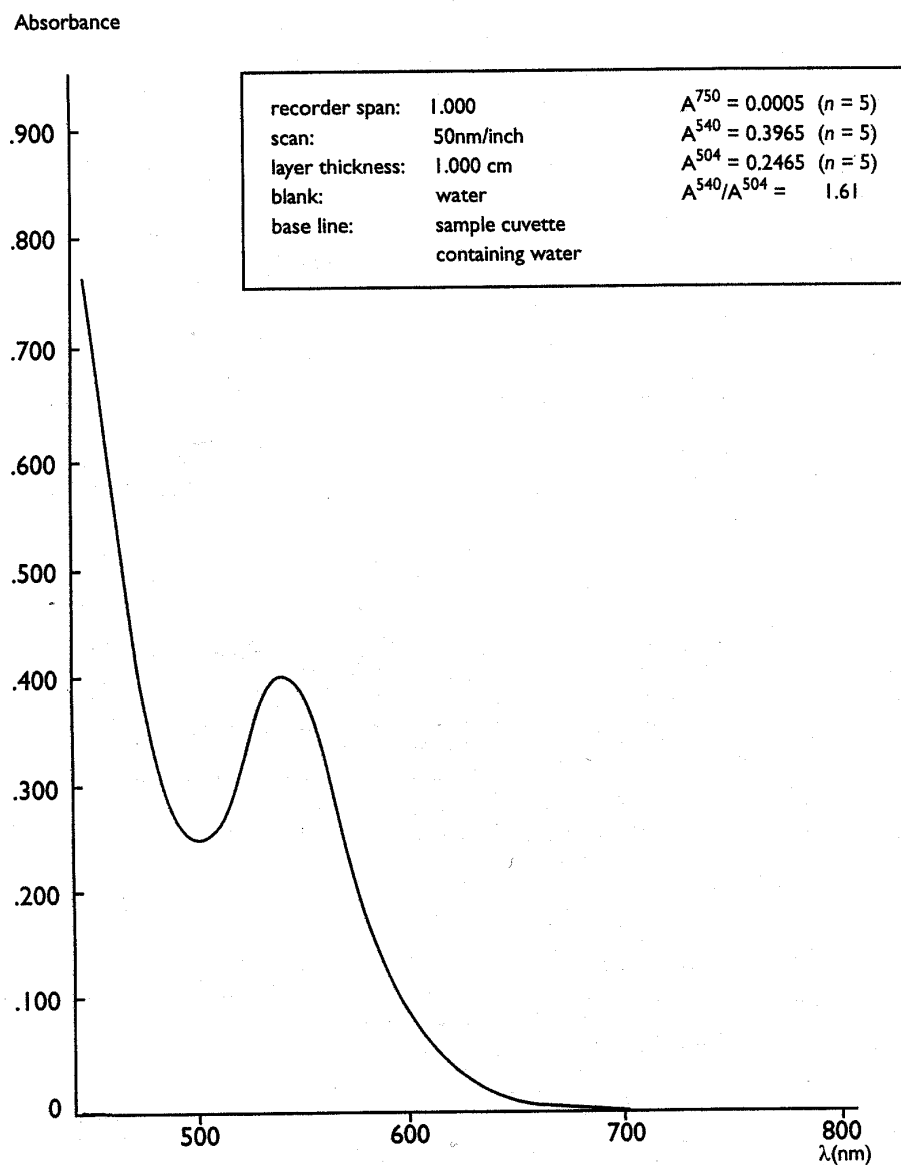
## 5 Test Principle of HiCN Method

In solution the ferrous ions ( $\text{Fe}^{2+}$ ) of the hemoglobins are oxidized to the ferric state ( $\text{Fe}^{3+}$ ) by potassium ferricyanide,  $\text{K}_3\text{Fe}(\text{CN})_6$ , to form hemiglobin, Hi. Hi, in turn, reacts with cyanide ions ( $\text{CN}^-$ ) provided by potassium cyanide, KCN, to form hemiglobincyanide, HiCN. The reaction is generally carried out at room temperature (18 to 25 °C), and the time necessary for full-color development is five minutes or less at a pH of 7.0 to 7.5.<sup>17,18</sup>



HiCN has a relatively broad absorption maximum around a wavelength ( $\lambda$ ) of 540 nm (see [Figure 1](#)).

The measuring instrument (see Section 6.4) is calibrated with a secondary HiCN standard (calibrator) (see Section 6.3 and Part II). The absorbance of HiCN at 540 nm is proportional to its concentration (Lambert-Beer's Law). Sample hemoglobin concentration is calculated from the measured absorbance (spectrophotometers, appropriately calibrated); is read directly from the instrument scale (direct reading photometers; hemoglobinometers); is read from a standard curve (photometers); or may be calculated from comparative measurements to a hemoglobin standard solution (photoelectric comparator).



**Figure 1. Absorption Curve International Hemoglobincyanide Reference Preparation (Batch 70600)**



## 6 Materials and Equipment

### 6.1 Glassware

Glassware used in the measurement of hemoglobins as HiCN must meet rigid standards of accuracy (Class A) and must be chemically clean.

Pipets required include the following:

- microliter or Sahli type pipets,  $20 \pm 0.1 \mu\text{L}$ ;
- measuring pipets, calibrated to deliver  $100 \pm 0.5 \mu\text{L}$  and  $0.5 \pm 0.005 \text{ mL}$ , or  $0.5 \pm 0.01 \text{ mL}$  graduated in 0.01-mL increments and/or positive displacement micropipets; and
- transfer pipets,  $5.0 \pm 0.005 \text{ mL}$ , and  $10 \pm 0.03 \text{ mL}$ .

**NOTE:** Calibration of positive displacement pipets should be performed with water and corrected for the temperature of the water, rather than with mercury because of differences in the meniscus between mercury and water or HiCN solutions. Because there is always a small amount of residual fluid present in the pipet after fluid delivery, this small amount (about 1%) of fluid should be included in the calibration. This is achieved by placing a small piece of tissue paper on the scale and using this to absorb the residual fluid from the piston tip. The balance's weighing chamber should be adequately humid to prevent evaporation, and weight measurements should be taken as soon as possible after completion of all fluid delivery.

Required glassware includes volumetric, Class A, calibrated glass flasks,  $25 \pm 0.03 \text{ mL}$ ,  $100 \pm 0.08 \text{ mL}$ ,  $250 \pm 0.12 \text{ mL}$ , and  $1000 \pm 0.3 \text{ mL}$ .<sup>19,20</sup>

In the routine method (see [Section 8.2](#)), 7- to 10-mL glass tubes and caps are used.

### 6.2 Reagents

#### 6.2.1 Composition

A suitable reagent was recommended by van Kampen and Zijlstra.<sup>17</sup>

KCN, (0.768 mmol/L)	0.050 g
K <sub>3</sub> Fe(CN) <sub>6</sub> , (0.607 mmol/L)	0.200 g
KH <sub>2</sub> PO <sub>4</sub> (Anhydrous), (1.029 mmol/L)	0.140 g
Nonionic Detergent	0.5 to 1.0 mL
Clinical Laboratory Reagent Water, Type 1	1000.0 mL

Clinical Laboratory Reagent Water, Type 1, meets the following specifications: microbiological content, less than ten colony-forming units per mL; resistivity, greater than  $10 \text{ m}\Omega \cdot \text{cm}$  at  $25^\circ\text{C}$ , measured in-line; silicate (SiO<sub>2</sub>) less than 0.05 mg/L. Only dry, analytic grade, anhydrous chemicals should be used. (Please see the current edition of NCCLS document [C3](#)— *Preparation and Testing of Reagent Water in the Clinical Laboratory*, for more information.)



### 6.2.2 Nonionic Detergents

The reagent recommended by van Kampen and Zijlstra<sup>17</sup> contained Sterox SE, an alkyl-phenol (thiol) polyethylene oxide; this detergent, however, is no longer available. Acceptable results have been obtained with Nonidet P-40 (registered trademark of Shell Oil Corporation), a polyethylene glycol P-ethyl/phenyl ether, and with Triton X-100 (registered trademark of Rohm and Haas), an octylphenylpolyethylene glycol ether.

### 6.2.3 Characteristics

The reagent is a clear, pale yellow solution that does not absorb light above 480 nm (see Figure 2). Some instrument-cuvette combinations have been found, however, to show apparent light absorption by the reagent between 500 and 600 nm [see Section 9.1(2)]. The pH is between 7.0 and 7.4; the osmolality, measured by means of freezing-point suppression, is 6 to 7 mOsm/kg.<sup>21</sup> Hemoglobins convert completely to HiCN within five minutes,<sup>17,18</sup> conversion of carboxyhemoglobin takes longer (up to 30 minutes for pure COHb); sulfhemoglobin converts to sulfhemiglobincyanide.<sup>22</sup> The reagent should be discarded if it turns turbid. The solution should be stored in an amber borosilicate glass bottle and tightly capped. The reagent is unstable if exposed to light. The reagent should be prepared fresh at least once a month.  $K_3Fe(CN)_6$  is destroyed by freezing<sup>23</sup>; decomposition can be prevented by the addition of, e.g., 20 mL ethanol per liter of reagent.<sup>24</sup>

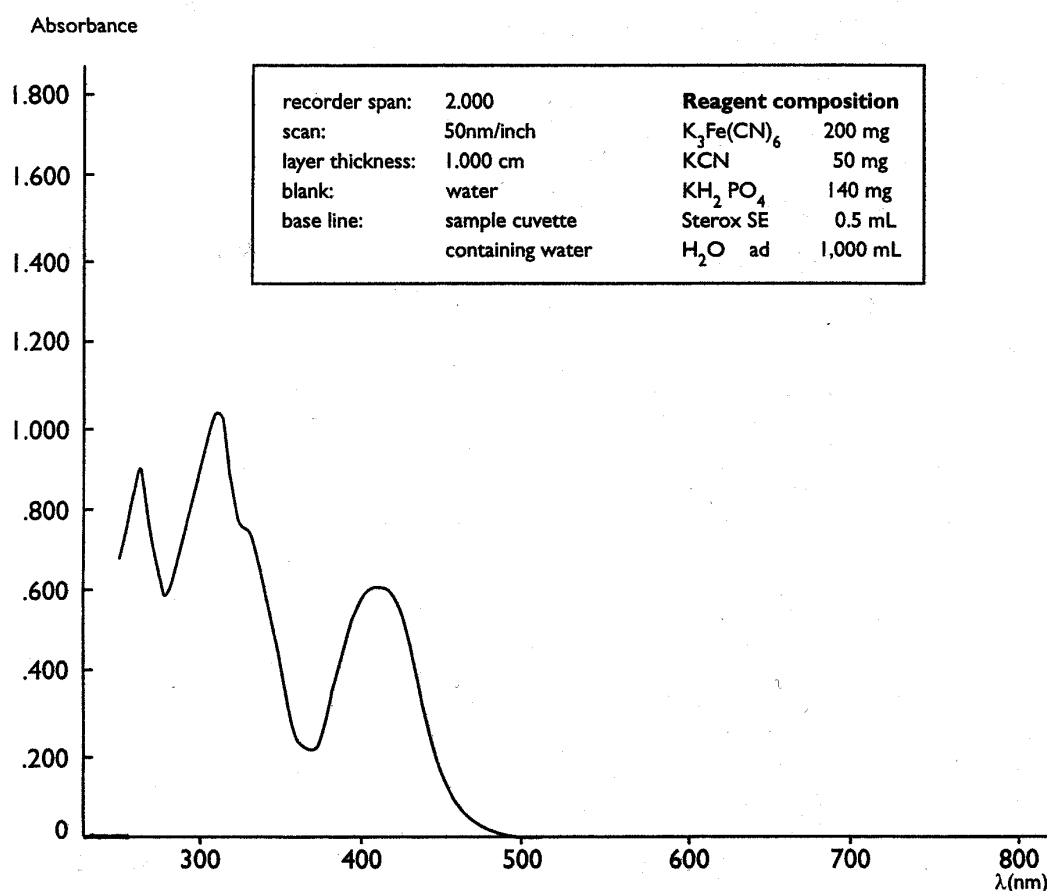


Figure 2. Absorption Curve Hemiglobincyanide-Method Reagent

### 6.2.4 Other Reagents

The original “Drabkin’s” reagent requires a conversion time of 15 minutes or more. It consists of NaHCO<sub>3</sub> (1.0 g), K<sub>3</sub>Fe(CN)<sub>6</sub> (200 mg), KCN (50 mg), and distilled water (to 1 L); it has a pH of 8.6.

“Modified Drabkin’s” reagent, without the NaHCO<sub>3</sub>, has a pH of 9.6 and an even longer conversion time. Because of the long conversion times, as well as the greater danger of turbidity of the resultant HiCN solutions, these “Drabkin’s” reagents are not recommended for use in the HiCN method.<sup>8</sup>

### 6.2.5 Precautions

The amount of potassium cyanide present in 1 L of reagent is appreciably less than the minimum lethal dose for a 70-kg human. However, hydrogen cyanide is liberated by acidification of the reagent. Therefore, dispose of solutions carefully and never allow them to come into contact with acids. Also check local ordinances for the disposal of cyanide-containing materials. Do not breathe fumes. For maximum safety, a fume hood should be used when preparing reagents, hands should be washed thoroughly immediately after handling chemicals, and food and drink should not be allowed in the vicinity. In case of contact with the reagent, flush the affected area with copious amounts of water. Get medical attention for the eyes.

If the dry reagent is ingested, call a physician immediately. Give frequent inhalations (one per minute) of amylnitrate or ammonia. Give artificial respiration if necessary.<sup>25</sup> If the person is conscious, induce vomiting.

Empty containers should be thoroughly rinsed before discarding them.

### 6.2.6 Interferences

In some pathological conditions (e.g., severe lipemia or proteinemia, white cell count > 20 x 10<sup>9</sup>/L, platelet count > 700 x 10<sup>9</sup>/L), turbidity of the resulting HiCN solutions may cause a significant overestimation of the hemoglobin concentration. To combat turbidity in such cases, reagents with increased ionic strength have been proposed.<sup>26</sup> It has, however, proven more practical to clear turbid HiCN solutions by membrane filtration (see Section 9.1).

Turbidity has also been encountered with blood specimens with a significant amount of hemoglobin S and severe microcytosis/hypochromasia.

## 6.3 Secondary Standards

Most HiCN standards contain approximately 500 to 800 mg/L (50 to 80 mg/dL) hemoglobincyanide. For HiCN methods employing a 250-fold dilution of blood samples, this provides an equivalent hemoglobin concentration of 125 to 200 g/L (12.5 to 20 g/dL). Only solutions in accordance with specifications as published by the International Council for Standardization in Haematology (ICSH) are acceptable.<sup>8</sup>

- (1) The absorbance of HiCN standards between 725 and 800 nm, e.g., at 750 nm, should be equal to or less than 0.003 per cm lightpath (absence-of-turbidity specification).<sup>9</sup>

$$A_{HiCn}^{750} \leq 0.003$$

- (2) The value of the absorbance of HiCN standards at 540 nm divided by the absorbance at 504 nm should lie between 1.59 and 1.63 (purity specification).<sup>9</sup>

$$1.59 \leq \frac{A_{\text{HiCN}}^{540}}{A_{\text{HiCN}}^{504}} \leq 1.63$$

- (3) The spectral absorbance curve of HiCN standards should conform to the shape of the spectral absorbance curve for a pure HiCN solution (purity specification). An example of a spectral absorbance curve of an (international) HiCN standard is shown in [Figure 1](#).
- (4) If an HiCN standard has been assayed to contain 800 mg/L (80 mg/dL) HiCN, the equivalent whole blood hemoglobin concentration is obtained by multiplying the stated HiCN content with the dilution factor used in processing the blood sample. For example, for a stated content of 800 mg/L (80 mg/dL) and the HiCN method dilution factor 251 times (0.02 mL of blood diluted with 5.0 mL), the equivalent Hb content is  $800 \cdot 251 = 200,800 \text{ mg/L} = 200.8 \text{ g/L}$  (20.08 g/dL).

## 6.4 Equipment

In general, (dual-beam) spectrophotometers are the most accurate instruments for the measurement of hemoglobin concentration. However, a variety of (spectro)photometers and colorimeters may be acceptable.

The hemoglobin concentration of an unknown sample is calculated from the absorbance value measured at 540 nm when a spectrophotometer is used [[see Section 10\(1\)](#)]. For accurate spectrophotometric measurements, the instrument spectral slit width should be less than 10% of the natural bandwidth of the assay chromophore.<sup>27</sup> When measuring HiCN solutions, accurate results are obtained with a spectral slit width of 6 nm or less. Most, if not all, modern (spectro)photometers will meet this requirement.

When a calibrated photometer, a photoelectric comparator, or a colorimeter is used, the hemoglobin concentration is:

- read directly from the instrument scale [[see Section 10\(3\)](#)];
- read from a standard curve [[see Section 10\(4\)](#)]; or
- compared to the measurement of a reference solution [[see Section 10\(5\)](#)].

To ensure the identification of instrument limitations, appropriate instrument manuals must be referred to before calibration.

Appropriate checks for linearity must be documented ([see Part II](#)).

## 7 Sample Collection and Preservation

### 7.1 Blood Samples

Although blood samples can be obtained through skin puncture (capillary blood), venipuncture is recommended. If capillary blood is used, samples must be appropriately collected. Venous blood should be anticoagulated with 3.7 to 5.4  $\mu\text{mol}$  EDTA (the elementary entity *mol* defined as the anhydrous molecule of ethylenediaminetetraacetic acid,  $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_8$ , CAS number 60-00-4, formula weight 292.24) per mL of blood; for dipotassium EDTA:anhydrous ( $\text{K}_2\text{EDTA}$ , CAS number 25102-12-9, formula weight 368.4) this equates to 1.4 to 2.0 mg/mL blood, for disodium EDTA:dihydrate  $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ , CAS number 6381-92-6, formula weight 372.23) to 1.4 to 2.0 mg/mL blood, and for tripotassium EDTA:

dihydrate ( $K_3EDTA \cdot 2H_2O$ , CAS number 65501-24-8, formula weight 442.5) to 1.6 to 2.4 mg/mL blood. The blood and anticoagulant must be mixed immediately. (Please refer to the current editions of NCCLS documents [H1](#)— *Evacuated Tubes and Additives for Blood Specimen Collection*; [H3](#)— *Procedure for the Collection of Diagnostic Blood Specimens by Venipuncture*; [H4](#)— *Procedures for the Collection of Diagnostic Blood Specimens by Skin Puncture*; and [M29](#)— *Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue* for more detailed information.)

Best results are obtained with fresh blood, and the test should be performed on the same day the specimen is drawn. It has, however, been shown that storing blood for one week at room temperature does not affect the hemoglobin value obtained with the reference procedure.

Once the sample and reagent are mixed, the resulting HiCN solution is stable and can be read when convenient. Storage for periods over six hours should be at 4 to 10 °C, in the dark, in tightly stoppered containers.

**NOTE:** When liquid  $K_3EDTA$ , 1.6 to 2.4 mg/mL is used the hemoglobin concentration may be up to 2.4% lower (evacuated tubes, 2 mL, draw 2.4%; 3 mL, draw 2.3%; 7 mL, draw 1.2%) because of dilution of the blood sample. (This also holds for other anticoagulants dispensed as solutions; the dilution factor must be calculated).

## 8 Dilution of Unknown Samples

Any convenient volume of blood and of reagent could be used. The resulting HiCN solution should, however, give a transmittance (T%) or absorbance (A) reading in the region where the resulting concentration error, for a given photometric error, is smallest, i.e.,  $T(\%) = 36.6$ ,  $A = 0.434$  (single-beam spectrophotometers). This means that for the range 120 to 160 g/L of hemoglobin (12 to 16 g/dL) the dilution of the blood sample should be between 200- and 250-fold. The concentration range that will give greatest accuracy in photometric analysis can be determined for any instrument by plotting a Ringbom curve.<sup>28</sup>

### 8.1 Reference Procedure

When the determination is to be used as a reference procedure, e.g., for instrument calibration purposes, a “macro” dilution is prepared.

- (1) Dilute 100  $\mu$ L (to-contain pipet) well-mixed blood and 25 mL (Class A volumetric flask) reagent to obtain a 251-fold dilution.
- (2) Alternatively, use 0.400- or 0.500-mL, Class A to-contain pipets and 100-mL Class A volumetric flasks to obtain a 200- to 250-fold dilution (calculate the appropriate dilution factor). **NOTE:** These “macro” dilutions virtually eliminate all variance caused by dilution errors.
- (3) Alternatively, use 0.04-mL, carefully calibrated, positive displacement micropipets and 10.0 mL of reagent. Calibration checks should be carried out before and after each series of reference hemoglobin measurements (see [Section 6.1](#)).

After aspirating blood, wipe the pipet in the upright position. Ensure that the meniscus at the pipet tip is flat. Deliver the contents of the pipet into the reagent and rinse the pipet by aspirating and delivering reagent at least five times. Reproducibility between different absorbance readings on separate dilutions from the same blood sample should be within 0.5% CV.

## 8.2 Routine Procedure, 251-Fold Dilution

- (1) Draw the sample into a 0.02-mL to-contain pipet (do not exceed 5 mm past the mark) at a slow and constant rate.

**NOTE:** Micropipets are filled by capillary action or by an artificially created vacuum. Disposable microliter pipets are available with a volumetric accuracy of  $\pm 1/4\%$ .

- (2) Wipe the outside of the pipet with damp (e.g., use water or physiologic saline) gauze. Be careful not to pull the specimen from the pipet by wiping across the tip of the pipet.
- (3) Adjust the amount of blood in the pipet to the mark.
- (4) Pull back the column of blood in the pipet to approximately 10 mm from the tip.
- (5) Rewipe the outer surface of the pipet with damp gauze.
- (6) Place the pipet into a tube containing 5.0 mL of reagent (see Section 6.2) so that the tip of the pipet is approximately 2.5 cm below the top of the solution. Tubes must be inspected before use to ensure they are free of dust.
- (7) Slowly expel the blood from the pipet. (It should settle to the bottom of the tube.)
- (8) Rinse the pipet eight to ten times with clean reagent from the top portion of the tube, being certain to rinse all portions of the pipet that have been in contact with the blood.
- (9) Cap the tube with an appropriate closure, stopper, or, e.g., Parafilm®, and mix five to six times by complete inversion.
- (10) Allow the diluted hemoglobin solution to remain at room temperature for at least five minutes to ensure full-color development.

## 9 Measurement of Unknown Sample

### 9.1 Reference Procedure

In practice, many HiCN solutions obtained when blood samples are diluted with reagent show some marginal turbidity ( $A^{750}$  up to 0.005) because of the presence of protein aggregates, microcrystals, and/or erythrocyte stromata remnants. Because an error of 0.001 in the absorbance measurement at 540 nm equates to a hemoglobin concentration error of 0.37 g/L (0.04 g/dL), the reference method requires membrane filtration of HiCN solutions before measuring. Centrifugation of HiCN solutions, even at high speeds, has proven to be inadequate in many cases.

- (1) Filter the HiCN solution using a syringe and a 25-mm diameter, low-binding, low-release membrane filter, 0.20- to 0.25- $\mu\text{m}$  mean pore diameter. Let the filtered solution stand for at least one minute to allow all air bubbles to escape.

**NOTE:** Excellent results are obtained with 25-mm, 0.22- $\mu\text{m}$  polyvinylidene difluoride Millex-GV (Millipore) and with 25-mm, 0.20- $\mu\text{m}$  polypropylene Puradisc 25 PP (Whatman) filters.<sup>29</sup> Any filter with equivalent properties can be used after the efficacy of the filtration step has been clearly demonstrated by serial filtrations of HiCN solutions and absorbance measurement at 750, 540, and 504 nm (see Section 6.3).

for purity and turbidity criteria of HiCN solutions). The reagent is filtered and measured at 540 and 750 nm to test for unwanted release from the membrane filter.

- (2) Using a calibrated spectrophotometer and matched glass 1.000-cm cuvettes, read the absorbance of the unknown, diluted sample (see Section 8.1) against the reagent as blank. (Single-beam spectrophotometers may require only one cuvette.)

**NOTE:** The reagent should not absorb light above 480 nm (see Section 6.2.3). In practice, however, some diode array and other instruments have been identified that show apparent light absorption by the reagent in the 500- to 600-nm range, with apparent absorbance values higher for matched quartz glass cuvettes than for optical glass cuvettes. It is not clear if apparent absorbance is caused by stray light or by internal cuvette reflections. It is therefore advisable to use a reagent blank. Water may be used as blank if the laboratory has shown that for a particular instrument-cuvette combination the reagent does not absorb light above 480 nm.

## 9.2 Routine Procedure

Using a calibrated (spectro)photometer and matched cuvettes, read the A or T(%) value of the unknown, diluted sample (see Section 8.1) against the reagent as blank.

**NOTE:** HiCN solutions that are slightly turbid,

$$A^{750} \geq 0.003, \frac{A^{540}}{A^{504}} \leq 1.59$$

[compare Sections 6.3(1) and 6.3(2)], can usually be purified by filtration through a membrane filter, mean pore diameter 0.20 to 0.25  $\mu\text{m}$ ; the first 0.5 mL of the filtrate should be discarded.<sup>30</sup> (Also compare Section 9.1.)

## 10 Determination of Corresponding Hemoglobin Concentration

- (1) When a spectrophotometer is used, calculate the corresponding hemoglobin concentration from the measured absorbance using the equation:

$$c \text{ (g / L)} = \frac{A_{\text{HiCN}}^{540} \cdot 16,114.5 \cdot F}{11.0 \cdot d \cdot 1,000}$$

where:

$c \text{ (g/L)}$  = hemoglobin concentration in gram per liter;

$A_{\text{HiCN}}^{540}$  = absorbance of the HiCN solution at 540 nm;

16,114.5 = relative molecular mass of the hemoglobin monomer (derived from 64,458/4)<sup>31</sup>;

F = dilution factor used (e.g., 251 for 0.02 mL of sample plus 5.0 mL of reagent);

$11.0 = \epsilon_{\text{HiCN}}^{540}$ , the quarter millimolar absorptivity of HiCN<sup>32</sup>;

$d$  = light path length, in centimeters, usually 1.000; and

1,000 = factor to convert milligram to gram.

- (2) For a dilution,  $F$ , of 1:251 and 1.000-cm cuvettes

$$c \text{ (g/L)} = 367.7 \cdot A_{\text{HiCN}}^{540}$$

- (3) When a direct reading photometer is used, read the corresponding hemoglobin concentration directly in g/L (mmol/L) from the instrument scale.
- (4) When a photometer is used, read the hemoglobin concentration corresponding to the T(%) or A value from the appropriate standard curve.
- (5) When a photometer or a photoelectric comparator is used to measure the unknown sample in comparison to a reference solution of known hemoglobin content, the hemoglobin content is calculated using the equation:

$$c_u \text{ (g/L)} = \frac{A_u^{540} \cdot c_r}{A_r^{540}}$$

where:

$c_u \text{ (g/L)}$  = hemoglobin concentration of unknown solution in gram per liter;

$A_u^{540}$  = absorbance of the unknown HiCN solution at 540 nm;

$c_r$  = hemoglobin concentration of reference solution in gram per liter; and

$A_r^{540}$  = absorbance of the reference HiCN solution at 540 nm.

## 11 Expression of Results

The International Union of Pure and Applied Chemistry (IUPAC) and the International Federation of Clinical Chemistry (IFCC) recommend that all clinical laboratory results (i.e., including hemoglobin) should preferably be reported in amount of substance, unit mole, with the appropriate prefix, per unit of volume liter (mmol/L). The International Council for Standardization in Haematology (ICSH) recommends that hemoglobin be reported as mass amount, unit kilogram, with the appropriate prefix, per unit of volume deciliter (g/dL). This recommendation was changed in 1976 to unit volume liter (g/L). The ICSH also stated that substance concentration (mmol/L) is acceptable, if desired, but that in such cases the elementary entity on the basis of which the mole is defined must be indicated on the report at all times (i.e., Hb or 4 Hb, Fe or 4 Fe), depending upon use of the hemoglobin monomer or the tetramer.<sup>33</sup>

This standard recommends reporting hemoglobin concentration as g/L, with g/dL permitted.

$$c(\text{g/dL}) = c(\text{g/L}) \cdot 0.1$$

If expression as substance concentration (mmol/L) is desired, the following factors, based on the hemoglobin monomer, should be used:

$$\begin{aligned} c(\text{mmol/L}) &= c(\text{g/L}) \cdot 0.0621 \\ &= c(\text{g/dL}) \cdot 0.621 \\ c(\text{g/dL}) &= c(\text{mmol/L}) \cdot 1.61 \\ c(\text{g/L}) &= c(\text{mmol/L}) \cdot 16.1 \end{aligned}$$



## Part II. Specifications for Secondary Hemiglobincyanide (HiCN) Standards

### 1 Introduction

Specifications for hemiglobincyanide (HiCN) standards were prepared by the International Council (previously Committee) for Standardization in Haematology (ICSH) in 1967 and revised in 1978, 1987 and 1996.<sup>7-9,34</sup> Properly prepared and properly stored HiCN solutions have been shown to be stable over long periods of time (up to 18 years).<sup>35-38</sup>

### 2 Scope

Part II describes specifications for, and characteristics of, hemiglobincyanide standards. This part includes:

- calculation of content from spectrophotometric measurement data;
- purity criteria; and
- sterility testing.

### 3 Secondary HiCN Standards

#### 3.1 Composition

HiCN standards are aqueous solutions of hemiglobincyanide in the range of 550 to 850 mg/L, which are free of debris. If human blood is used to prepare the solutions, it must be tested for the absence of hepatitis and for the absence of antibodies to HIV. The HiCN content in mg/L (mg/dL) must be given within a tolerance of  $\pm 0.5\%$ .

#### 3.2 Preparation of International HiCN Standards

For guidance purposes, ICSH/WHO international HiCN standards are prepared as follows:<sup>39</sup>

- (1) Fresh or outdated human blood is obtained, and the erythrocytes are washed twice with sodium chloride, 9 g/L (0.154 mol/L), then twice with sodium chloride, 12 g/L (0.205 mol/L).
- (2) Add to the red cell mass a volume of toluene 0.4 times the volume of the red cell mass and a volume of distilled water equal to the volume of the red cell mass. Thoroughly stir this mixture and place it at 4 to 6 °C for approximately 12 hours.

**NOTE:** In place of toluene, carbon tetrachloride ( $\text{CCl}_4$ ) has been used effectively. When  $\text{CCl}_4$  is used, the refrigeration step is not necessary. *CAUTION: These organic solvents are toxic and potentially carcinogenic when inhaled. Do not pipet by mouth. Use of a fume hood is recommended.*

- (3) The refrigerated mixture is centrifuged and the upper (toluene) layer removed by suction. The bottom (hemoglobin) layer is decanted from under the more-or-less-solid middle layer of packed cell stromata.

- (4) The hemoglobin solution is filtered through ash-free filter paper or through a sintered glass filter to remove any remaining cellular debris, and the hemoglobin concentration of the solution is determined. Usually a concentration of about 150 g/L (15 g/dL) is obtained.
- (5) A calculated amount of hemoglobin solution is added to a reagent containing 200 mg  $K_3Fe(CN)_6$ , 50 mg KCN, and 1.0 g  $NaHCO_3$  per liter to obtain a solution containing HiCN in the range of 550 to 600 mg/L (55 to 60 mg/dL). Note that the reagent does not contain a nonionic detergent (cell stroma and plasma proteins have been removed) and that the pH has not been brought to the 7.0 to 7.5 range (no time constraints on the reaction).
- (6) The HiCN solution is filtered, under pressure, through a filter of 0.25 to 0.45  $\mu m$  mean pore size for sterilization purposes, and dispensed, under aseptic conditions, as 10-mL aliquots.
- (7) The HiCN content of the International Haemoglobinocyanide Reference Preparation is determined in at least five different laboratories and is certified to contain the stated value  $\pm 1$  mg ( $\pm 0.2\%$ ), e.g.,  $580 \pm 1$  mg HiCN per liter.

### 3.3 Spectrophotometric Characteristics of HiCN Standards

#### 3.3.1 Content

Using an appropriate blank,  $A_{HiCN}^{540}$  is measured on a narrow-band spectrophotometer, the wavelength and absorbance scales of which have been appropriately calibrated,<sup>40,41</sup> and in which the absence of stray light has been verified. The slitwidth of the instrument is chosen so that the half-intensity bandwidth at the nominal wavelength of measurement is  $\leq 2$  nm (compare to Section 3.3.2(3) NOTE). The cuvettes in which the calibration standards are measured are plane parallel with an inner wall-to-wall distance of 1.000 cm (tolerance 0.5%). Measurements are carried out between 20 and 25 °C.

The HiCN content is calculated from the absorbance measurement at 540 nm with the following equation:

$$c \text{ (mg / L)} = \frac{A_{HiCN}^{540} \cdot 16,114.5}{11.0 \cdot d}$$

where:

$c$  = HiCN content in milligram per liter;

$A_{HiCN}^{540}$  = absorbance of the HiCN solution at 540 nm;

16,114.5 = relative molecular mass of the hemoglobin monomer (derived from 64,458/4);<sup>31</sup>

11.0 =  $\epsilon_{HiCN}^{540}$ , the quarter millimolar absorptivity of HiCN at 540 nm;<sup>32</sup> and

$d$  = light path length in centimeters, to be known with an accuracy to three decimal places.  
(Usually,  $d = 1.000 \pm 0.005$ .)

For a 1.000-cm light path length:

$$c \text{ (mg/L)} = 1,465 \cdot \frac{A_{\text{HiCN}}^{540}}{A_{\text{HiCN}}^{504}}$$

### 3.3.2 Purity

The purity of HiCN calibrators is controlled by the following actions:

- (1) Comparing the shape of the absorbance spectrum between 450 and 750 nm to the shape of the spectrum of the International Haemoglobinocyanide (HiCN) Standard (Figure 1).

**NOTE:** The International HiCN Standard is available, free of charge, to manufacturers and distributors of secondary HiCN calibrators through “National Holders.” As of July 2000, there is no National Holder in the United States.

Information on the ICSH International HiCN Standard may be obtained from the ICSH Executive Secretary, 13 Buchanan Street, Milngavie, Glasgow F62 8AW, Scotland, UK.

- (2) Determining the value of the quotient  $\frac{A_{\text{HiCN}}^{540}}{A_{\text{HiCN}}^{504}}$ ; the value must lie between 1.59 and 1.63.<sup>34</sup>
- (3) Measuring in the infrared, above 710 nm (e.g., at 750 nm), to check for turbidity.

$A_{\text{HiCN}}^{750}$  must be less than or equal to 0.003 per cm of light path.<sup>9</sup>

**NOTE:** The spectrophotometer used must be sufficiently sensitive to detect the presence of small particles in low concentration. Excellent sensitivity is obtained when measuring with a half-intensity bandwidth of 0.7 nm or less at 750 nm. The upper limits of acceptable bandwidth have not yet been determined.

### 3.4 Sterility

The contents of an appropriate number of vials of secondary HiCN standard should be inoculated in/on aerobic and anaerobic media with incubation at 22 °C and 37 °C to ensure sterility.<sup>39</sup>

### 3.5 Label

The label must include the producer’s name; the batch (lot) number; the HiCN content [mg/L, mg/dL; mean and standard deviation (SD)]; the value of the (equivalent) hemoglobin content (mean and standard deviation); an expiration date considered within safe limits and supported by stability testing data; and conditions for storage. The producer must perform continuing control of stability and notify all customers if a certain batch (lot) no longer meets requirements.

### 3.6 Certification

Manufacturers of secondary HiCN standards can have their products certified as “meeting ICSH specifications” by “National Holders.” However, as of July 2000 the United States “National Holder” has discontinued its certification program.

### 3.7 Storage

HiCN standards stored at 4 to 8 °C in the dark are stable over long periods of time. Dispensed into amber borosilicate glass ampoules and sealed, stability has been demonstrated for periods of about 15 years.<sup>42</sup> HiCN standards must not, however, be allowed to become frozen. There is no published stability data for standards dispensed into glass screwcap vials.

There is very little data available on the stability of HiCN solutions stored at temperatures other than 4 to 8 °C. Accelerated degradation studies have indicated no deterioration of HiCN solutions during the first six months when stored at 20 to 22 °C and a decrease of  $A_{\text{HiCN}}^{540}$  of up to 0.5% per month when kept at 37 °C.<sup>42</sup>

## 4 Other HiCN Reference Solutions

There is insufficient published documentation of the stability and reproducibility of spectral characteristics of HiCN solutions outside the 550- to 850-mg/L HiCN content range. Although many such solutions are available for the purpose of checking photometers and the preparation of standard curves, such solutions cannot, at this time, be designated or certified as secondary HiCN calibrators “that meet ICSH specifications.”

## 5 Alternative Source Material

It has been shown that measurements of  $\epsilon_{\text{HiCN}}^{540}$  in various animal species give virtually identical values as those obtained for human blood.<sup>43-45</sup> Some years ago 14 bovine specimens were measured to determine hemoglobin iron resulting in (n = 92)  $\epsilon_{\text{HiCN}}^{540} = 10.96$ , standard error of the mean 0.008.<sup>46</sup> Because the relative molecular mass of bovine hemoglobin has been reported to be 64,533 for the tetramer,<sup>47</sup> the HiCN concentration of bovine HiCN standards can be accurately calculated from absorbance measurements at 540 nm. Preliminary accelerated degradation data indicate that bovine HiCN solutions are at least as stable as those made from human source material.<sup>46</sup>

## Appendix. Protocol for Evaluation of Routine Methods for the Determination of Hemoglobin Against the Reference Method

The literature contains many examples of user product evaluations with many different experimental and statistical procedures for comparing two methods that measure the same analyte. This appendix will help the laboratorian with the independent establishment of a test system's performance characteristics within the user's particular environment. More detailed information may be found in publications by ICSH,<sup>48</sup> IFCC,<sup>49</sup> and NCCLS document [EP9](#)— *Method Comparison and Bias Estimation Using Patient Samples*.

### Technical Assessment Using Patient Samples

The method that is subject to the evaluation is referred to as the “test method”; the reference method is described in this document.

- (1) At least 40 fresh human blood specimens are collected over at least five operating days. Follow manufacturer's instructions for specimen collection for the test method; the reference method requires EDTA-anticoagulated venous specimens. The test method should be evaluated over the clinically meaningful range. Therefore, 15% of specimens should be equal to or less than 90 g Hb per liter; 25% should contain from 91 to 120 g/L; 50% should contain from 121 to 170 g/L; and 10% should contain more than 171 g/L hemoglobin.
- (2) For a given specimen, analysis by the test method and by the reference method should occur within two hours of each other, whether the specimens are freshly drawn or stored. Analyze all specimens in duplicate with the test method and the reference method. Run the duplicate determinations in reverse order compared to the first analysis and randomize the concentration in the sequence.
- (3) Precision should be measured on replicate measurements (ten, if possible) of a specimen from each quartile. The precision of the test method and the reference method should be determined in a similar manner for comparative purposes.
- (4) Make two plots of the data. The first is a scatterplot of the means of the duplicates of the test method versus those of the reference method; the second is a bias plot where the difference between the mean of each duplicate test determination and the mean of each reference determination is plotted against the mean of the reference duplicate determination. These plots will allow a visual linearity check, a visual outlier check, and a visual precision constancy check. For further statistical analyses see NCCLS document [EP9](#)— *Method Comparison and Bias Estimation Using Patient Samples*.

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## Additional Reference

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**NCCLS consensus procedures include an appeals process that is described in detail in Section 9.0 of the Administrative Procedures. For further information, contact the Executive Offices or visit our website at [www.nccls.org](http://www.nccls.org).**

## Summary of Comments and Subcommittee Responses

H15-A2: *Reference and Selected Procedures for the Quantitative Determination of Hemoglobin in Blood; Approved Standard—Second Edition*

### General

1. The procedures described in the H15 standard are not used in clinical laboratories. The standard seems most useful to manufacturers of calibrators.
  - **The working group does not agree. The procedures described in H15 are required for whole blood calibration procedures of automated hematology analyzers, are necessary in the evaluation of instruments and alternative methods for the determination of hemoglobin concentration, and should be applied (see Bull BS, Hay KL. In *Methods in Hematology – Quality Control*. I. Cavill, 2<sup>nd</sup> ed. New York: Churchill Livingstone; 1990; 172-192) when patient red cell measurements are used for calibration and control of hematology analyzers. Also see Section 3.**
2. Our quality assurance reference laboratory recently revised its reference procedure to coincide with H15-A2, *Reference and Selected Procedures for the Quantitative Determination of Hemoglobin in Blood—Second Edition; Approved Standard*. The procedure includes a filtering which we had not performed previously. The recommended procedure was followed. Filtered and unfiltered dilutions were measured for comparison and the hemoglobin concentration, in mg/dL, was derived from a standard curve. The diluted standard solutions were not filtered. A bias was seen in the control results as well as in the donated, fresh blood (this may be due to the fact that assay assignment for hemoglobin on the controls originated from instrument calibration based on the previous reference method which did not include filtration). We were anticipating slightly lower absorbance readings; however, we feel the absorbances obtained may be too low. Should the certified reference have been filtered?
  - **Certified reference material (standards) should not need filtration, as “certification” should ensure that the material is nonturbid ( $A^{750} \leq 0.003$ ), consists of pure HiCN ( $1.59 \leq A^{540} / A^{504} \leq 1.63$ ), and has an accurate assigned value. If a laboratory believes inaccurate values are being obtained, the laboratory should verify the absence of turbidity and the purity of the standard by measuring the absorption at 750, 540, and 504 nm. Whole blood specimens are filtered after dilution with reagent to ensure the absence of turbidity. Also see Sections 6.3 and 9.1 in Part I, and Section 3.3.2 in Part II.**
3. What effect would commercial cyanmethemoglobin reagent have on this procedure? The reagent we employ closely meets the reference requirements and is a component of the CAP-certified/ICSH-specified cyanmethemoglobin standard.
  - **Commercial HiCN reagents should have no effect on this procedure. This can be verified by measuring a fresh whole blood sample and measuring the absorbance at 750, 540, and 504 nm. The values found for  $A^{750}$  and  $A^{540} / A^{504}$  should approximate the values described in Section 9.1 of Part I.**

4. Taking into account that our controls may have a bias, if another manufacturer's control product is used and recovery within the assay range is not achieved, how do we proceed with troubleshooting?
  - **See responses to Comments 2 and 3.**
5. Should performance be assessed using the international HiCN reference solution? How can the solution be obtained?
  - **The WHO/ICSH international HiCN standard is released only to national standardizing organizations, and to designated laboratories where there is no national standards organization, for the purpose of certifying secondary national or commercial HiCN standards.**
6. van Kampen-Zijlstra reagent may yield turbidity in patients exhibiting high gammaglobulinemia. It has been suggested that the ionic strength of the reagent be increased (*Clin Chim Acta*. 1979; 93:163-164). Please note, the ICSH method for determination of the "secondary standard" is not the problem. It is difficult to apply the method to pathological samples due to potentially unbalanced concentrations and components of plasma proteins. However, the potential of increased turbidity with the van Kampen-Zijlstra reagent should be mentioned in the selected procedures to the reference materials and standards.
  - **Pathological specimens may indeed result in turbid solutions after dilution with the reagent. Such solutions may, however, be "cleared" by membrane filtration. To clarify this issue, a section on interference has been added to Part I, Section 6.2: Reagents.**

## Section 8.2

7. Section 8.2(9) states, "Tubes are capped with Parafilm "M" and mixed." Any tube capped with parafilm can leak, or solution droplets can adhere to the film after inversion. Another method of mixing should be used, such as gentle shaking or slow vortexing.
  - **Part I, Section 8.2(9) of H15-A2 states "Cap the tube with, e.g., (for example) Parafilm.®" This section has been revised to clarify the closure process.**

## **Part II**

### Section 1

8. The introductory statement for this section indicates that "properly prepared and properly stored HiCN solutions have been shown to be stable over long periods of time (up to 18 years)." I am unable to identify a section in the document that indicates a recommended "proper storage" condition for the secondary standard solution. ICSH (reference 8) indicates a storage temperature of 4° C. Typical refrigerator temperatures for clinical laboratory purposes are targeted at temperatures between 4 and 8° C. We believe the storage conditions should be recommended within this protocol to assure stability of secondary standards. We have been able to demonstrate quite clearly that freezing these secondary standards destroys their properties. This too should be indicated as interference if improper storage conditions are not followed.
  - **The commentor is quite correct that "freezing of these secondary standards destroys their properties." Also compare Part I, Section 6.2.3. A new section on storage of the standards has been added to the document.**

## Summary of Delegate Comments and Committee Responses

H15-A3: *Reference and Selected Procedures for the Quantitative Determination of Hemoglobin in Blood; Approved Standard—Third Edition*

### Part I

#### General

1. We recommend discussion of reagents used by latest generation analyzers (e.g., TOA Sysmex, Abbott CellDyn) where cyanide-free lysing reagent is used for determination of hemoglobin. This would provide a standard for analyzer companies.
- **NCCLS document H15-A3 describes the determination of hemoglobin concentration in human blood by the HiCN (cyanmethemoglobin) method (Section 3, Scope). Cyanide-free reagents thus, are outside the scope of this document. Also, see Section 1, Introduction: “If any other method is used in routine measurement (...) it should be adjusted to obtain comparability with the haemiglobincyanide method” (references 7 and 8).**
2. This standard would be very useful for the manufacturers of instruments and commercial controls/calibrators, but would not be practical in most clinical hematology labs.
- **The working group does not agree. The reference method is required for whole blood calibration procedures of analyzers. It is necessary to assign values to (in-house) control materials and is applicable when red cell measurement means are used for calibration or control of analyzers.**

#### Section 7.1

3. Paragraph 1 states, "Venous blood should be anticoagulated with 1.5 to 2.2 mg EDTA (dipotassium or disodium salt; 3.7 to 5.4 pmol) per mL of blood and mixed immediately."

The tripotassium salt of EDTA in the range of 1.2 to 2 mg EDTA per mL of blood is also an acceptable anticoagulant. Also, the pmol range cannot be the same for elements of different molecular weight; therefore, this paragraph should be changed. We recommend it be revised using ISO 6710 (1<sup>st</sup> edition 1995-08-01, Single-use containers for venous blood specimen collection) as a guide and reference. Annex E of this international standard (copy enclosed) provides for each additive, including salts of EDTA, the nominal amount within a range that shall be used.

- **The working group agrees that the elementary entity “mol of EDTA” should be defined. The paragraph has been revised to include the CAS numbers of the various EDTA salts, the degree of hydration, and the mass amounts to be used. The working group, however, retains the molar concentration requirement as 3.7 to 5.4 μmol per mL blood. (For additional information, please refer to NCCLS document H1—*Evacuated Tubes and Additives for Blood Specimen Collection*; ICSH Expert Panel on Cytometry, Recommendations of the International Council for Standardization in Haematology for ethylenediaminetetraacetic acid anticoagulation of blood for blood cell counting and counting and sizing. *Am J Clin Path.* 1993;100:371-372.)**

4. The “NOTE:” on page 11 states that “When K3EDTA... is used, the hemoglobin content may be up to 1.0% lower because of dilution of the blood sample.”

We recommend the note be deleted, as the comment is not specific to K3EDTA. It is also relevant to K2EDTA and Na2EDTA since these additives may be present in blood collection tubes in liquid form. Also, a dilution factor of up to 1.0% is probably not significant.

- **The working group agrees that, for any anticoagulant dispensed in liquid form, there is a dilution effect that should be considered. A note to this end has been added. However, the working group does not agree that a dilution factor of up to 1% is not significant. In a reference method, any inaccuracy has significance and must be identified.**

## Part II

### References

5. Reference 7: The year listed in this citation (i.e., 1997) is incorrect.
  - **The typographical error has been corrected. The citation has been corrected to read as follows:**  
**International Committee for Standardization in Haematology. Recommendations for reference method for haemoglobinometry in human blood (ICSH Standard EP 6/2: 1977) and specifications for international haemiglobincyanide reference preparation (ICSH Standard EP 6/3: 1977). *J Clin Path.* 1978;31(2):139-143.**
6. Reference 10: The authors of this reference are incorrect. The correct authorship is B.S. Bull and K.L. Hay.
  - **The authorship has been corrected. The citation has been corrected to read as follows:**  
**Bull BS, Hay KL. Intralaboratory quality control using patients' data. In: Cavill I, ed. *Quality Control*. 2<sup>nd</sup> ed. Edinburgh, Scotland: Churchill Livingstone; 1990;172-192.**

## Related NCCLS Publications\*

- C3-A3**      **Preparation and Testing of Reagent Water in the Clinical Laboratory; Approved Guideline—Third Edition (1997).** This document provides guidelines on water purified for clinical laboratory use; methods for monitoring water quality and testing for specific contaminants; and water system design considerations.
- C46-P**      **Blood Gas and pH Analysis and Related Measurements; Proposed Guideline (2000).** This document provides clear definitions of the several quantities in current use, and provides a single source of information on appropriate specimen collection, preanalytical variables, calibration, and quality control for blood pH and gas analysis and related measurements.
- EP9-A**      **Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline (1995).** This document addresses procedures for determining the relative bias between two clinical methods or devices, and for the design of a method comparison experiment using split patient samples and analysis of the data.
- H1-A4**      **Evacuated Tubes and Additives for Blood Specimen Collection—Fourth Edition; Approved Standard (1996).** This standard includes requirements for blood collection tubes and additives including heparin, EDTA, and sodium citrate.
- H3-A4**      **Procedure for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard—Fourth Edition (1998).** This document provides procedures for the collection of diagnostic specimens by venipuncture, including line draws, blood culture collection, and venipuncture in children. Recommendations for the order of draw are also included.
- H4-A4**      **Procedures for the Collection of Diagnostic Blood Specimens by Skin Puncture; Approved Standard—Fourth Edition (1999).** This standard provides detailed descriptions and explanations of proper collection techniques, as well as hazards to patients due to inappropriate specimen collection by skin puncture procedures.
- H7-A3**      **Procedure for Determining Packed Cell Volume by the Microhematocrit Method; Approved Standard—Third Edition (2000).** This document describes a standard microhematocrit method for determining packed cell volume; specifications for recommended materials and information on potential sources of error are also included.
- H18-A2**      **Procedures for the Handling and Processing of Blood Specimens; Approved Guideline—Second Edition (1999).** This document includes criteria for preparing an optimal serum or plasma sample and for the devices used to process blood specimens.

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

**Related NCCLS Publications (Continued)**

- M29-A**      **Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Approved Guideline (1997).** This document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting. Specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and recommendations for the management of blood-borne exposure are also included.
- NRSCL8-A**   **Terminology and Definitions for Use in NCCLS Documents; Approved Standard (1998).** This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory (NRSCL).

## NOTES



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