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

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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.

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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.”¹ 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.²

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 hemoglobinocyanide (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 hemoglobinocyanide (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:³⁻⁵ 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).⁶ 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

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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 1978,⁷ 1987,⁸ and 1996.⁹ 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 haemiglobinazide; 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.^{7,8}

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^{10,11}; 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^a

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

Absorbance (symbol A), *n* - The logarithm of the ratio of radiant power (I_o) incident on the sample to the radiant power (I) transmitted by the sample.¹²

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

Alternative terms sometimes used are “extinction” and “optical density.”¹³ 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.,

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

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.”¹³

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; HiN₃ versus N₃metHb.

Hemiglobincyanide (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.”^{7,13}

Hemoglobins, *n* - All those hemoglobin derivatives normally present in circulating blood. They include deoxyhemoglobin (HHb), oxyhemoglobin (O₂Hb), carboxyhemoglobin (COHb), and hemiglobin [Hi;

^a Some of these definitions are found in NCCLS document NRSL8—*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.

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.

* 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).