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Determination of Serum Iron, Total Iron-Binding Capacity and Percent Transferrin Saturation; Approved Standard

This document provides methods for determining serum iron and total iron-binding capacity; and describes the measurement of serum iron concentration as well as the determination of the percent saturation of transferrin with iron.

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Determination of Serum Iron, Total Iron-Binding Capacity and Percent Transferrin Saturation; Approved Standard

Abstract

This document describes the measurement of serum iron concentration, provides guidelines for the determination of serum total iron-binding capacity, and describes the determination of the percent saturation of transferrin with iron. The methods are linear over a wide range of iron concentrations, interference is negligible, and the precision is adequate. Although the methods, as described, are tedious and demand a large sample size and meticulous processing, they can be (semi)automated and the required amount of sample can be decreased. An example of automation is included. The document also provides reference ranges by age groups, race, and gender for serum iron concentration, total iron-binding capacity, and % transferrin saturation as determined during the U.S. National Health and Nutrition Examination Surveys, 1971-74, 1976-80, and 1988-94.

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Determination of Serum Iron, Total Iron-Binding Capacity and Percent Transferrin Saturation; Approved Standard

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Onno W. van Assendelft, M.D., Ph.D., Chairholder
Basil T. Doumas, Ph.D.
Virgil F. Fairbanks, M.D.
Elaine W. Gunter, M.T.(ASCP)
Daniel A. Nealon, Ph.D.

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Jean C. Joseph, Ph.D.	St. Mary Medical Center Long Beach, California
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Gary L. Myers, Ph.D.	Centers for Disease Control and Prevention Atlanta, Georgia
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Luc Van Hove, M.D., Ph.D.	Abbott Laboratories Santa Clara, California
Onno W. van Assendelft, M.D., Ph.D.	Centers for Disease Control and Prevention Atlanta, Georgia

Working Group on Iron

Onno W. van Assendelft, M.D., Ph.D. **Chairholder**	**Centers for Disease Control and Prevention** **Atlanta, Georgia**
Basil T. Doumas, Ph.D.	Medical College of Wisconsin Milwaukee, Wisconsin
Virgil F. Fairbanks, M.D.	Mayo Clinic Rochester, Minnesota
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Foreword

Human metabolic requirements for iron are met by a positive balance during the years of growth and by a rate of absorption that is physiologically matched with a relatively fixed rate of loss in the adult. Iron balance is unusual in that there is no physiological route for the excretion of excessive amounts and the avoidance of iron loading depends on the regulation of iron absorption. Many factors may influence iron absorption. Under physiologic conditions, however, only three factors appear to be important: the amount of iron ingested, its bioavailability, and the iron status of the patient. Absorption of iron is primarily controlled by the mucosal cells of the proximal small intestine, with a fraction of available iron absorbed inversely proportional to the body iron stores.[1, 2] Iron deficiency occurs when the dietary intake of bioavailable iron is inadequate, when absorption is impaired, or when bleeding occurs causing iron losses that exceed the capacity of the gastrointestinal tract to extract iron from the diet. Iron (over)loading may result from an abnormal increase in the amount of iron absorbed, from the parenteral administration of iron, or from blood transfusion.

Storage iron occurs predominantly in two forms: as a soluble component of ferritin which is composed of a soluble (FeOOH)x core within an apoferritin protein shell, or as part of insoluble hemosiderin. There is evidence that the most recently formed hemosiderin has the most rapidly available iron for mobilization from storage to transport pools.[3] Under conditions of iron overloading, hemosiderin iron increases relative to ferritin iron with massive deposits in parenchymal cells leading to tissue and organ damage.

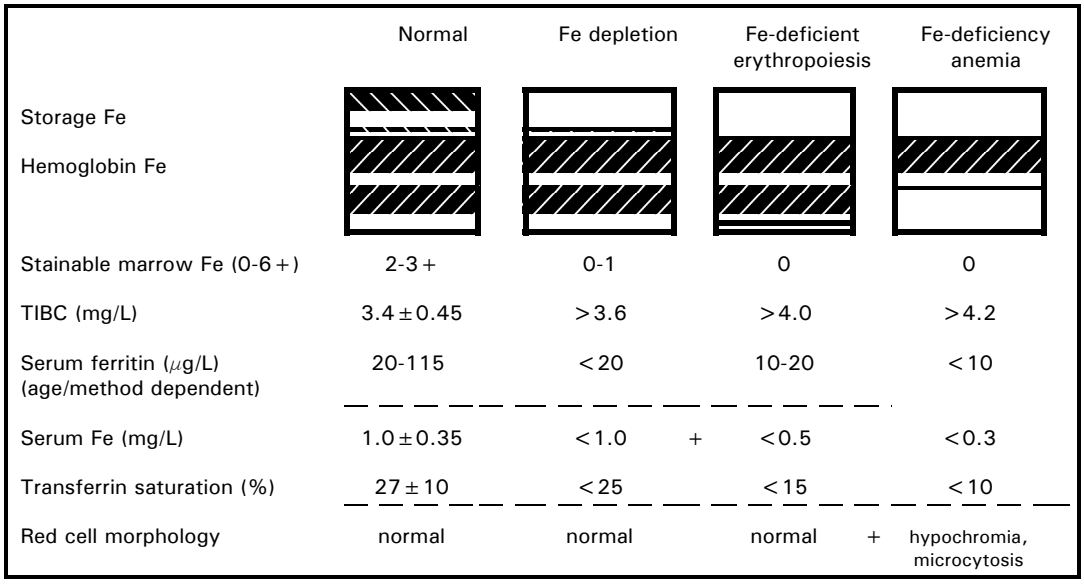
Mammals have specific iron-binding proteins that move iron from sites of absorption and storage to sites of use. The best characterized of these proteins are the transferrins: two-sided single-chain iron-binding proteins widely distributed in physiologic fluids and cells. Two major types have been identified: serum transferrin and lactoferrin. Serum transferrin is the major carrier protein of iron in blood and tissues[4]; lactoferrins are found predominantly in tissue fluids and cells and seem to function in the body's defense against infection as well as in iron transport.[5] Transferrin has a relative molecular mass of approximately 79 600[6] and is a major serum protein, usually one-third saturated with iron, corresponding to a plasma iron concentration of 1 mg/L (17.9 μmol/L;100 μg/dL)

Iron deficiency is the most common nutritional deficiency in both developing and developed countries. Iron deficiency results from a persisting negative iron balance due to inability to meet physiologic needs through diet. Iron deficiency not only causes anemia, but may have an effect on immunocompetence,[7,8] and has been associated with behavioral abnormalities and reduced intellectual performance in children.[9,10] In older children and adults, iron deficiency usually implies chronic blood loss.

The first, mildest stage of iron deficiency is iron depletion in which iron stores (*e.g.,* reticuloendothelial cells of liver, spleen, and bone marrow) are substantially reduced. In the second stage, iron stores are depleted but anemia is not yet demonstrable. The third and most severe degree of iron deficiency involves frank anemia, frequently with hypochromic and microcytic erythrocytes.

Plasma or serum ferritin concentration has proven a useful index of storage iron.[11] Direct evidence relating serum ferritin concentration to the size of the body iron stores has been obtained by comparison with chemical measurements of non-heme iron concentration in bone marrow,[1] and by studies in which the body iron store was estimated by phlebotomy-induced blood loss.[12-14] Although the serum ferritin assay provides a useful and convenient way of detecting an absence of storage iron in uncomplicated iron deficiency, its usefulness is limited in many clinical situations in which iron deficiency coexists with infection and other inflammatory disorders, or with neoplasms, liver disease, or chronic renal disease. However, in these conditions, the usefulness of serum iron assays may also be quite limited.

Foreword (Continued)



	Normal	Fe depletion	Fe-deficient erythropoiesis	Fe-deficiency anemia
Stainable marrow Fe (0-6+)	2-3+	0-1	0	0
TIBC (mg/L)	3.4±0.45	>3.6	>4.0	>4.2
Serum ferritin (μg/L) (age/method dependent)	20-115	<20	10-20	<10
Serum Fe (mg/L)	1.0±0.35	<1.0	<0.5	<0.3
Transferrin saturation (%)	27±10	<25	<15	<10
Red cell morphology	normal	normal	normal	hypochromia, microcytosis

Laboratory test results during gradual development of iron deficiency (Modified from Bothwell et al. *Iron Metabolism in Man.* Oxford: Blackwell Scientific; 1979).

Once iron stores are depleted, the rate of delivery of iron to the bone marrow is limited and the serum iron concentration falls, as does the percent saturation of transferrin. On the other hand, the total iron binding capacity, TIBC, which is a measure of both unsaturated and saturated transferrin, is already affected before the iron supply fails: TIBC rises as storage iron decreases.[15,16] The reliability of transferrin saturation, calculated as serum iron concentration/TIBC, as an index of iron deficiency is, however, limited by variability of the serum iron concentration, particularly because of marked diurnal variation. Even when specimens are collected at the same time of day, variability in the same person on different days may be as much as 40%.[17]

The most prevalent form of iron overload occurs as an inherited, nonhematological disorder of iron overabsorption: (idiopathic) hereditary hemochromatosis. In the early stages of the disease a number of screening tests may be helpful. Serum iron concentration is usually high and the circulating transferrin is usually completely or almost completely saturated; the TIBC of serum is usually reduced. Serum ferritin may be increased, although this is not invariably the case early in the iron-loading process. In clinical practice the best screening procedure for iron overload thus involves combined measurements of serum iron concentration, TIBC, and calculation of the percent transferrin saturation. In 1996 an Iron Overload Expert Advisory Panel to the Centers for Disease Control and Prevention recommended that determination of % transferrin saturation be used as a screening test for (idiopathic) hereditary hemochromatosis.[18]

Key Words

Hemochromatosis, iron deficiency, iron overload, percent transferrin saturation, (serum) iron, (serum) iron-binding capacity, TIBC, transferrin

Determination of Serum Iron, Total Iron-Binding Capacity and Percent Transferrin Saturation; Approved Standard

1 Introduction

The measurement of serum iron, total iron-binding capacity (TIBC), and calculation of the percent transferrin saturation have long been used as diagnostic procedures in the evaluation of iron deficiency. However, these tests are relatively insensitive for this purpose. A relatively sensitive test for the absence of storage iron in uncomplicated iron deficiency is the determination of serum ferritin, although the usefulness of this test is also limited, especially in situations where iron deficiency coexists with infections or inflammatory disorders. Calculation of the percent transferrin iron saturation, however, is a sensitive screening procedure for iron overload.

Laboratory errors in serum iron assays caused by iron contamination were common in the past, but have been largely eliminated by the use of disposable plastic supplies. The International Council for Standardization in Haematology, ICSH, has published recommendations for the determination of serum iron and TIBC.[19-23] The methods, however, remained subject to interference by copper and influenced slightly by heme iron,[24] and sources of variability in the determination of TIBC included the concentration of the saturating iron solution and the type and amount of magnesium used to remove the unbound iron.[25]

Modifications developed at the Centers for Disease Control and Prevention, CDC, to maximize the sensitivity of the method and minimize interference included replacing thioglycollic acid by ascorbic acid as reducing agent, adding thiourea to the chromogen reagent,[26-28] use of basic instead of "light" magnesium carbonate,[26] and a dilution factor of 3 instead of 2 in the determination of TIBC to bring the absorbance values in the same range as those of the serum iron determination.

The CDC-NCCLS-modified method has been compared to the original ICSH reference method and the results have shown to be comparable, if not identical;[29] and the method has been used in the evaluation of other, (semi)automated methods.[30,31] The methods have also been automated by CDC and used extensively during the National Health and Nutrition Examination Surveys (NHANES) I (1971-74), II (1976-80) and Hispanic (1982-84), without thiourea added to the chromogen reagent,[26] and NHANES III (1988-94), with thiourea added to the chromogen reagent.[32] (See Appendix A.)

2 Scope

This standard describes the recommended method for the determination of serum iron, guidelines for the determination of TIBC, and the calculation of % transferrin iron saturation. Accurate determinations are required:

- for the evaluation of patient iron status and the differential diagnosis of anemia;
- for screening and diagnosis of iron overload syndromes; and
- in the evaluation of other manual or automated methods for the determination of these analytes.

This standard is thus intended primarily for clinical laboratory personnel.

3 Units of Measurement

Serum iron and TIBC values can be expressed as mass concentration, unit μg/L, mg/L, or as substance concentration, unit μmol/L.

The International Federation of Clinical Chemistry, IFCC, and the International Union of Pure and Applied Chemistry, IUPAC, recommend the exclusive use of the liter as unit of volume when reporting laboratory results using the Système International d'Unités(SI).[33]

Although many published values are given per deciliter (dL; 10^{-1}L) as the unit of volume, this document, in conformance with ICSH, IFCC, IUPAC, and World Association of Societies of Pathology, (WASP), will use the liter and, where applicable, give both mass and substance concentration.

Conversion factors:

μmol/L	x	55.85	= μg/L; x 0.05586 = mg/L
μmol/L	x	5.585	= μg/dL
μg/L	x	0.1	= μg/dL
μg/L	x	0.017905	= μmol/L
mg/L	x	17.905	= μmol/L
μg/dL	x	10	= μg/L
μg/dL	x	0.17905	= μmol/L

4 Specimens

The recommended method for the determination of serum iron requires a 2 mL sample; the determination of TIBC requires 1 mL. The methods have been successfully "miniaturized" with respect to sample requirements.[26,29,32]

Serum or **heparinized** plasma is suitable,[34] and may be either fresh or frozen. Serum iron and TIBC are stable and specimens may be stored (-20 °C or lower); serum iron levels are not affected by freeze-thaw cycles, TIBC values tend to decrease after two freeze-thaw cycles.[35]

Specimens anticoagulated with ethylene-diaminetetraacetic acid (EDTA) salts, with oxalate or with citrate are not suitable because these anticoagulants also chelate iron, thus preventing its reaction with the chromogen used in the iron determination. Grossly hemolyzed specimens are not suitable because of the potential contribution of iron from the hemoglobin.

For best results, fasting samples should be obtained. However, there is a marked diurnal variation of serum iron concentration (see Section 6.7.6). Even when specimens are collected at the same time of the day, the within-person day-to-day variability may be as high as 40%.[17]

5 Standard Precautions

Because it is often impossible to know which might be infectious, all patient blood specimens are to be treated with standard precautions. Specimens from any patient could be infected with human immunodeficiency virus, HIV, or hepatitis B or C virus, HBV, HCV. Proper blood collection techniques should be followed to minimize risk to the laboratory staff, and gloves should be worn when appropriate. For specific precuations for preventing the laboratory transmission of bloodborne infection from laboratory instruments and materials; and recommendations for the management of bloodborne exosure, refer to NCCLS document M29—*Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*.

6 Recommended Method for the Determination of Serum Iron

6.1 Principle of the Method

Addition of acid to serum releases iron from transferrin (by lowering the pH) and precipitates of serum proteins. The iron, Fe(III), in the supernatant is reduced to Fe(II) and determined quantitatively by photometric measurement of the absorbance of the colored complex formed between Fe(II) and FerroZine™ (or Ferene®) as chromogen. Thiourea is added to the chromogen reagent to complex copper [Cu(II)] which can also bind to FerroZine™ and yield falsely elevated iron values.

6.2 Materials

6.2.1 Instrumentation

- Spectrophotometer, total bandwidth around 560 nm less than 8 nm; absorbance repeatability over the range A = 0.000 to 1.000 not to exceed 0.001; zero drift not to exceed 0.001 absorbance unit per hour
- Centrifuge
- Vortex mixer
- Cellulose acetate, or similar, membrane filter, 0.45 μm mean pore diameter

6.2.2 Chemicals

- Deionized water
- FerroZine™ iron reagent; 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid) -1,2,4-triazine, monosodium, monohydrate ($C_{20}H_{14}N_4O_6S_2Na{\bullet}H_2O$). See Figure 1 for spectral characteristics. Alternatively, Ferene® may be used. See Section 6.8.
- Hydrochloric acid (HCl)
- Iron wire (Fe), 99.9% purity
- L-ascorbic acid ($C_6H_8O_6$)
- Sodium acetate, trihydrate ($C_2H_3O_2Na_3{\bullet}3H_2O$)
- Thiourea (CH_4N_2S)
- Trichloroacetic acid (CCl_3COOH).

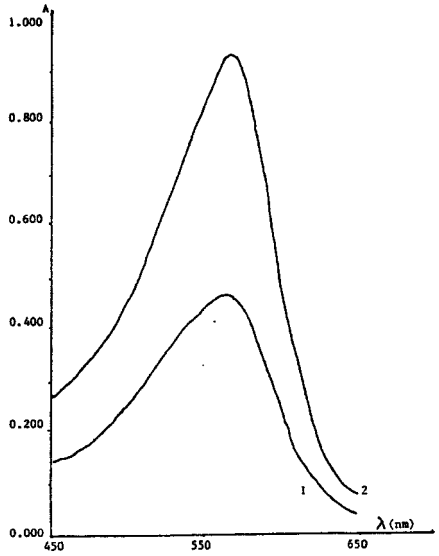


Figure 1. Absorbance curve of ferrozine-iron complex. Ferrozine 100 mg/L, Fe (II) 0.5 mg/L (1) and 1.0 mg/L (2). Molar absorptivity of this complex is reported to be 27 900 LA $cm^{-1}A$ mol^{-1}.[41] (Data provided by Hach Company, Ames, Iowa).

6.2.3 Glassware

All glassware used must be iron-free: it is washed in a warm solution of a nonionic detergent, rinsed with deionized water, soaked in dilute nitric acid (0.75 mol/L), rinsed with deionized water, and dried in a dust-free environment.

- Centrifuge tubes, conical, graduated, 15-mL capacity, with caps
- (Centrifuge) tubes 13 x 100 mm, disposable, iron-free, with caps
- (Centrifuge) tubes, 15 x 125 mm, disposable, iron-free, with caps
- Flasks, volumetric, Class A, with stoppers, 200 mL, 500 mL, 1 000 mL. (For Class A flask volume tolerances, see ASTM[a] E 288, E 542, and E 694 standards)
- Pipets, volumetric, Class A, 1.0 mL, 2.0 mL, 4.0 mL, 6.0 mL, 8.0 mL, 10.0 mL. (For Class A volumetric pipet tolerances, see ASTM E 969-93 standard)

Lots of disposable laboratory ware should be screened for iron contamination. Two mL of precipitating-reducing reagent (Section 6.3.5) and 1 mL of chromogen reagent (Section 6.3.6) are added to each of a representative sample of tubes. They are mixed and allowed to stand for 30 minutes. Gross iron contamination can be detected with the naked eye; trace iron contamination is checked by spectrophotometric measurement: the absorbance at 562 nm, must be less than 0.015.

6.3 Reagents

6.3.1 Trichloroacetic Acid, 50% (w/v)

Dissolve 250.0 g trichloroacetic acid in deionized water and dilute to 500 mL in a volumetric flask.

The reagent is stable for at least 6 months when stored at 4 °C.

WARNING: ***Use fume hood.***

6.3.2 Acetate Solution, 4 mol/L

(1) Dissolve 544.4 g sodium acetate trihydrate in deionized water.

(2) Dilute to 1.0 L in a volumetric flask and filter the acetate solution through a cellulose acetate membrane filter, 0.45 μm mean pore diameter.

The solution is stable for one month when stored at ambient (room, 18 - 25 °C) temperature.

6.3.3 Hydrochloric Acid, 6 mol/L

Add 500 mL concentrated hydrochloric acid to 250 mL deionized water in a volumetric flask; dilute to 1.0 L.

WARNING: ***Take extreme care; use proper protective clothing; and use a fume hood.***

6.3.4 Hydrochloric Acid, 0.1 mol/L

Add 8.3 mL concentrated hydrochloric acid to 500 mL deionized water in a volumetric flask; dilute to 1.0 L.

WARNING: ***Take extreme care; use proper protective clothing; and use a fume hood.***

[a]ASTM: American Society for Testing and Materials

6.3.5 Precipitating-Reducing Reagent

(1) Dissolve 5.0 g L-ascorbic acid in 200 mL 50% (w/v) trichloroacetic acid (Section 6.3.1).

(2) Add 33 mL, 6 mol/L hydrochloric acid (Section 6.3.3). Adjust to volume with deionized water in a 1.0 L volumetric flask. The reagent is stable for one week when stored at 4 °C.

Because of the relatively short shelf life, a smaller quantity (*e.g.*, 100 to 250 mL) may be prepared.

Warning: ***Prepare the precipitating-reducing reagent in a fume hood.***

6.3.6 Chromogen Reagent

(1) Dissolve 525 mg FerroZine™ and 15.0 g thiourea in 4 mol/L acetate solution (Section 6.3.2) and adjust to volume in a 1.0 L volumetric flask.

(2) Store reagent overnight at 4 °C before use.

The reagent is stable for one week when stored at 4 °C. Because of the relatively short shelf life, a smaller quantity (e.g., 100 to 250 mL) may be prepared. See Section 6.8 for ferene as alternative chromogen.

6.4 Iron Standard Solutions

6.4.1 Stock Iron Standard Solution, 1.000 g Fe per L

(1) Dissolve 1.000 g iron wire, > 99.9% purity (e.g., National Institute of Standards and Technology SRM 937) in 12 mL of concentrated hydrochloric acid in a 1.0 L volumetric flask.
(2) Warm slightly.
(3) After complete dissolution, cool flask to ambient (room; 18 - 25 °C) temperature and dilute to volume with deionized water.

WARNING: ***Take extreme care; use proper protective clothing; and use a fume hood.***

The solution is stable indefinitely when stored at ambient (room; 18 - 25 °C) temperature in a well-stoppered container to prevent evaporation.

6.4.2 Intermediate Iron Standard Solution, 50 mg Fe per L

Dilute 25 mL of iron stock solution (Section 6.4.1) to 500 mL in a volumetric flask with hydrochloric acid, 0.1 mol/L (Section 6.3.4). The solution is stable for at least three months when stored at ambient (room; 18 - 25 °C) temperature in a well-stoppered container to prevent evaporation.

6.4.3 Iron Standard Working Solutions

Transfer 0, 1.0, 2.0, 4.0, 6.0, 8.0, and 12.0 mL Intermediate Iron Standard Solution, 50 mg Fe/L (Section 6.4.2) into a corresponding series of 200-mL volumetric flasks and dilute to volume with 0.1 mol/L hydrochloric acid (Section 6.3.4). A final Fe concentration of 0 (reagent blank), 250, 500, 1 000, 1 500, 2 000, and 3 000 µg/L, respectively (0, 25, 50, 100, 150, 200, and 300 µg/dL) is thus obtained. The solutions are stable for at least three months when stored at ambient (room;18 - 25 °C) temperature.

6.5 Determination of Serum Iron Concentration

(1) Mix serum or heparinized[34] plasma specimen thoroughly.
(2) Pipet 2.0 mL of each serum/plasma sample, or iron standard working solution (Section 6.4.3) into labeled 15-mL centrifuge tubes.
(3) Pipet 2.0 mL of precipitating-reducing reagent (Section 6.3.5) into each tube and mix well using a vortex mixer.
(4) Let stand for at least 15 minutes, then centrifuge at about 1 500 x *g* for 15 minutes.
(5) Transfer 2.0 mL of the supernatant to labeled 13 x 100 mm tubes and add 1.0 mL of the chromogen reagent (Section 6.3.6). Mix well using a vortex mixer.
(6) Centrifuge tubes at about 1 100 x *g* for 15 min and transfer the supernatants to clean, labeled tubes.
(7) Measure the absorbance of the final reaction mixtures with a spectro-

photometer at 562 nm using 1.000 cm cuvettes and the reagent blank/precipitating-reducing solution (1:1) as blank.

(8) Analyze all iron standard working solutions and patient samples in duplicate.

(9) Prepare a calibration curve from the measured absorbances of the iron standard working solutions and read the unknown samples from the curve, or calculate the results by means of linear regression.

The method may be "miniaturized" as long as the serum: precipitating-reducing-reagent: chromogen-reagent remains 2:2:1;[26, 32, 36] in this case semi-micro spectrophotometer cuvettes, compatible with the measuring instrument, will be required. The method has been automated successfully.[28, 37] Automated pipetting devices may be used, provided they are free of iron contamination.

6.6 Quality Control

6.6.1 Three levels of quality control serum pools should be analyzed, in duplicate, with every standard curve and with every batch of specimens analyzed. Because lyophilization may result in significant amounts of easily split-off hemoglobin iron which is then also detected in the serum iron assay, the use of frozen or liquid quality control materials is recommended. However, excellent precision has been obtained with freeze-dried materials. If use of lyophilized materials is contemplated, the laboratory must first verify that assigned values are recovered accurately and that acceptable precision is obtained for materials with, or for materials without, assigned values.

6.6.2 The preparation of suitable quality control material is given in Appendix B.

6.6.3 Control data, collected over several years, of commercial and in-house quality control preparations from three laboratories have been collected by the NCCLS Area Committee on Clinical Chemistry and Toxicology, Working Group on Iron.

Table 1. Serum Iron Control Data Collected over Several Years by Three Laboratories for Various In-house and Commercial Control Materials.

Laboratory A

level	n	mean mg/L	S.D. mg/L	C.V. %
1*	18	0.61	0.022	3.6
2*	18	1.77	0.030	1.7
3*	18	2.95	0.025	0.9
1**	36	0.60	0.023	3.8
2**	36	1.82	0.026	1.4
3**	36	3.07	0.033	1.1
1†	22	1.18	0.018	1.5
2†	22	2.47	0.025	1.0
1††	24	0.80	0.021	2.6
2††	24	2.61	0.036	1.4

* = commercial control #1; ** = commercial control #2
† = commercial control #3; †† = commercial control #4

Laboratory B

level	n	mean mg/L	S.D. mg/L	C.V. %
1	102	0.698	0.028	2.8
2	102	0.881	0.033	2.9
3	102	1.383	0.032	4.6
1	33	0.738	0.021	2.9
2	33	0.982	0.026	2.6
3	33	1.433	0.028	1.95

= in-house preparation #1; = in-house preparation #2

Laboratory C

level	n	mean mg/L	S(T) = S.D.¶ mg/L	S(wr)¶ mg/L	C.V. %
1§	40	0.500	0.025	0.015	5.0
2§	40	1.013	0.028	0.018	2.8
3§	40	2.513	0.051	0.046	2.0
4§	40	3.006	0.073	0.066	2.4
1§§	40	1.009	0.052	0.020	5.1
2§§	40	2.431	0.089	0.089	3.8

§ = commercial control #5; §§ = commercial control #6

¶ Within run variance S(wr) and total imprecision S(T) (=S.D.) calculated from duplicate determinations, one run per day, over 20 days, according to protocol described in NCCLS document EP5—*Precision Performance of Clinical Chemistry Devices.*

6.7 Interferences and Sources of Error

6.7.1 Contamination

A major source of error may be contamination of glassware and disposable materials (see section 6.2.3).

6.7.2 Hemoglobin Iron

A second source of error is inclusion of (some of the) hemoglobin iron in the measurement of serum iron:

- grossly hemolyzed specimens are unacceptable.
- precautions should be taken to minimize hemolysis when drawing the specimen. (See also NCCLS document *H3 Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture*.)
- in the recommended method, hemoglobin iron interference is minimized by the use of ascorbic acid as reducing agent.

6.7.3 Copper and Zinc

The colorimetric determination of iron is subject to interference by copper and zinc.[28, 38-42] In the recommended method copper and zinc interference is significantly reduced by the inclusion of thiourea in the chromogen reagent[27, 28, 32] because these elements are avidly chelated by thiourea.

6.7.4 Control Materials

Lyophilized specimens and control materials are generally not suitable because the acid reagent may liberate iron from lyophilized hemoglobin in reconstituted serum.[21, 23] They are, however, suitable if values are assigned after rehydration of the lyophilized product. If use of lyophilized materials is contemplated, the laboratory must first verify that assigned values are recovered accurately and that acceptable precision is obtained for materials with, or for materials without, assigned values.

6.7.5 Plasma

When plasma samples are to be used for the determination of serum iron, heparin must be used as an anticoagulant (See Section 4).

6.7.6 Diurnal Variation

In the interpretation of serum iron (and TIBC, and percent transferrin iron saturation) results, reports of marked diurnal variation must be taken into account.[43-48]

6.8 Alternative Chromogen

FerroZine™ has been characterized as having a molar absorptivity of 27 900 [41] to 28 600 $L \cdot cm^{-1} \cdot mol^{-1}$ [49] with best results in the pH range of 3 to 6. In 1984 Hennesy et al.[50] described Ferene®, the disodium salt of 3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazine ($C_{16}H_8N_4O_8S_2Na_2 \cdot H_2O$) as an attractive, cheaper alternative to FerroZine for the determination of serum iron. Ferene® has a molar absorptivity of 34 500 $L \cdot cm^{-1} \cdot mol^{-1}$, with most reliable results also in the pH range of 3 to 6.

6.8.1 Ferene® Chromogen Reagent

Dissolve 600 mg Ferene® and 15.0 g thiourea in 4 mol/L acetate solution (Section 6.3.2) and adjust to volume in a 1.0 L volumetric flask. The reagent is stable for one week when stored at 4 °C. Measure the absorbance at 593 nm. Because of the relatively short shelf life, a smaller quantity (e.g. 100 to 250 mL) may be prepared.

6.8.2 Control Data

With Ferene® as chromogen, quality control materials gave a CV of 3.3%, 1.8%, and 1.7% at, respectively, 794 µg/L, 2.044 and 5.208 mg/L iron (D.A. Nealon, unpublished results; personal communication).

6.9 Reference Range

Values for adult males are generally higher than those for adult females; in children values are lower than in adults.

Values, as published, include:

females: 60 - 180 µg/dL (600 - 1 800 µg/L; 10.7 - 32.2 µmol/L) [51-53]
males: 70 - 180 µg/dL (700 - 1 800 µg/L; 12.5 - 32.2 µmol/L) [51, 53]

110 - 180 μg/dL (1 100 - 1 800 μg/L; 19.7 - 32.2 μmol/L) [52]

Serum iron values found in the National Health and Nutrition Examination Surveys, NHANES I (1971-74) and NHANES II (1976-80) were determined without the addition of thiourea to the chromogen reagent. The values by age and sex[54, 55] have been corrected for an estimated copper interference[24] in the method. An average correction of 9.65% was determined by measuring 101 specimens in parallel with one chromogen reagent containing thiourea, and one without thiourea added.

Table 2. Serum Iron Values, NHANES I (1971-74) and NHANES II (1976-80)

Female

		mean			percentile ranges					
age group (yrs)	n	μg/L	μg/dL	μmol/L	5th μg/L	95th μg/L	5th μg/dL	95th μg/dL	5th μmol/L	95th μmol/L
2-11	2530	790	79	14.1	310	1 350	31	135	5.6	24.2
12-17	1450	900	90	16.1	410	1 490	41	149	7.3	26.7
18-44	6160	900	90	16.1	400	1 610	40	161	7.2	28.8
45-74	5500	850	90	15.2	420	1 530	42	153	7.5	27.4

Male

2-11	2675	800	80	14.3	300	1 400	30	140	5.4	25.1
12-17	1570	950	95	17.0	500	1 670	50	167	9.0	29.9
18-44	4080	1000	100	17.9	540	1 620	54	162	9.7	29.0
45-74	4940	920	92	16.5	470	1 550	47	155	8.4	27.8

Some differences were found between the black and the white population.

- In black females, age 12 - 74 years, serum iron values were 5-10% lower than those in white females of the same age group.
- In black males, age 45 - 74 years, mean serum iron was about 15% lower than in white males of the same age group.

Preliminary data from NHANES III (1988-94) by age, gender, and race are given in Appendix A.

7 Guideline for the Determination of Total Iron Binding Capacity of Serum, TIBC

7.1 Principle of the Method

Iron is added to an aliquot of serum to fully saturate the transferrin iron-binding sites; the excess, unbound iron is removed. Addition of acid then releases the bound iron from transferrin and precipitates the serum proteins. The iron in the supernatant is reduced to Fe(II) and determined quantitatively by photometric measurement of the colored complex formed between Fe(II) and FerroZine™ (or Ferene®) as chromogen. The chromogen reagent contains thiourea to complex Cu(II).

Because of the sample preparation step (saturation with iron; removal of unbound iron), some investigators have proposed determining the unsaturated (latent) iron binding capacity of serum, UIBC; TIBC then represents the sum of

UIBC and serum iron concentration. UIBC has been determined using radioactive iron, assuming that no exchange will take place between radio-iron added and iron previously present,[23, 56] and by saturating serum with Fe(III), removing the unbound iron excess, complexing the reduced Fe(II) with a chromogen, then measuring spectrophotometrically.[57] In certain automated chemistry analyzers a precisely known amount of iron in solution is added to diluted serum and the amount of iron that did not bind to transferrin is measured colorimetrically; UIBC is then calculated as the difference in color produced by the original amount of added iron and the color produced by the residual, unbound, iron in solution.[58] Relatively large, apparently method-specific differences have been reported.[18,59,60]

7.2 Materials

7.2.1 Instrumentation (also see Section 6.2.1)

- Spectrophotometer, total bandwidth < 8 nm around 560 nm;
- centrifuge;
- vortex mixer;
- cellulose acetate membrane filter, 0.45 μm mean pore diameter.

7.2.2 Chemicals (also see Section 6.2.2)

- Basic magnesium carbonate, approximate formula $(MgCO_3)_4 \bullet Mg(OH)_2 \bullet 5H_2O$;
- deionized water;
- FerroZine™ Iron Reagent (alternative Ferene; see Section 6.8);
- hydrochloric acid;
- iron wire, > 99.9% purity;
- L-ascorbic acid;
- sodium acetate, trihydrate;
- thiourea;
- trichloroacetic acid.

7.2.3 Glassware (also see Section 6.2.3)

- graduated, 15-mL capacity conical centrifuge tubes, with caps;
- disposable 13 x 100 mm and 15 x 125 mm, iron-free (centrifuge) tubes, with caps;
- volumetric, Class A flasks with stoppers, 200-, 500-, and 1 000-mL capacity;
- volumetric, Class A pipets, 1.0-, 2.0-, 4.0-, 6.0-, 8.0-, and 10.0-mL capacity.

All glassware used must be iron-free; glassware may be washed in a warm solution of nonionic detergent, rinsed with deionized water, soaked in dilute (0.75 mol/L) nitric acid, rinsed with deionized water, and dried in a dust-free environment. Manufacturer's lots of disposable laboratory ware should be screened for iron contamination (See Section 6.2.3).

.

7.3 Reagents

Also see Sections 6.3 and 6.4.

7.3.1 Iron Saturating Solution, 2 mg/L; alternatively 4 mg/L

Dilute 1.0 mL (alternatively 2.0 mL) stock iron solution, 1.000 g/L (Section 6.4.1) to 500 mL with deionized water, in a volumetric flask. The solution is stable indefinitely when stored at ambient (room; 18 - 25 °C) temperature in a well-stoppered container to prevent evaporation.

7.3.2 Trichloroacetic Acid, 50% (w/v)

Dissolve 250.0 g trichloroacetic acid in deionized water and dilute to 500 mL. Shelflife > 6 months, stored at 4 °C.

WARNING: ***Use fume hood.***

7.3.3 Acetate Solution, 4 mol/L

Dissolve 544.4 g sodium acetate trihydrate in deionized water and dilute to 1.0 L. Filter the acetate solution through a 0.45 μm mean pore diameter cellulose acetate membrane filter. Shelflife 1 month, stored at ambient (room: 18-25 °C) temperature.

7.3.4 Hydrochloric Acid, 6 mol/L

Add 500 mL concentrated hydrochloric acid to 250 mL deionized water and dilute to 1.0 L.

WARNING: ***Take extreme care; use fume hood.***

7.3.5 Hydrochloric Acid, 0.1 mol/L

Add 8.3 mL concentrated hydrochloric acid to 500 mL deionized water and dilute to 1.0 L.

WARNING: ***Take extreme care; use fume hood.***

7.3.6 Precipitating-Reducing Reagent

Dissolve 5.0 g L-ascorbic acid in 200 mL 50% (w/v) trichloroacetic acid (7.3.2) and 33 mL 6 mol/L hydrochloric acid (See Section 7.3.3); dilute to 1.0 L. Shelflife 1 week, stored at 4 °C.

WARNING: ***Use fume hood.***

7.3.7 Chromogen Reagent

Dissolve 525 mg FerroZine™ (alternatively 600 mg Ferene) and 15.0 g thiourea in 4 mol/L acetate solution (see Section 7.3.3) and dilute to 1.0 L. Store overnight at 4 °C before use. Shelflife 1 week, stored at 4 °C.

7.3.8 Iron Standard Working Solutions

Transfer 0, 1.0, 2.0, 4.0, 6.0, 8.0, and 12.0 mL Intermediate Iron Standard Solution, 50 mg Fe/L (Section 6.4.2) into a series of 200-mL volumetric flasks and dilute to volume with 0.1 mol/L hydrochloric acid (7.3.5). The final iron concentration will be 0 (reagent blank), 250, 500, 1 000, 1 500, 2 000, and 3 000 μg/L (0, 25, 50, 100, 150, 200, and 300 μg/dL).

The working standard solutions are stable for > 3 months when stored at ambient (room: 18-25 °C) temperature.

7.4 Determination of Total Iron Binding Capacity of Serum, TIBC

(1) If frozen, thaw and mix serum or heparinized plasma[34] thoroughly.

(2) Pipet 1.0 mL of each serum/plasma sample into labeled 15 x 125 mm tubes.

(3) Add 2.0 mL of iron saturating solution, 2 mg/L (Section 7.3.1) to each tube and mix well with a vortex mixer: allow to stand for approximately 15 min at ambient (room; 18 - 25 °C) temperature. Specimens with low serum iron concentration, i.e., < 200 μg/L (20 μg/dL; 3.6 μmol/L) may require 2.0 mL of iron saturating solution, 4 mg/L (Section 7.3.1).

(4) Add 300 (+/- 50) mg basic magnesium carbonate (Section 7.2.2) to each tube of diluted serum and immediately mix well using the vortex mixer. Repeat after 15 minutes and again after 30 minutes, using the vortex mixer.

(5) Allow to stand at ambient (room; 18 - 25 °C) temperature for at least another 15 minutes, then centrifuge at > 1 000 x *g* for 15 minutes.

(6) Transfer 2.0 mL of the supernatant, without disturbing the sediment, to labeled 15-mL centrifuge tubes. Also pipet 2.0 mL of each of the iron standard working solutions (Section 7.3.8) into labeled 15-mL centrifuge tubes.

(7) Pipet 2.0 mL of precipitating-reducing reagent (Section 7.3.6) into each tube and mix well using a vortex mixer; let stand for at least 15 minutes, then centrifuge at about 1 500 x *g* for 15 minutes.

(8) Transfer 2.0 mL of the supernatant to labeled 13 x 100 mm tubes and add 1.0 mL of the chromogen reagent (Section 7.3.7); mix well using a vortex mixer.

(9) Centrifuge tubes at about 1 100 x *g* for 15 minutes and transfer the supernatants to clean, labeled tubes.

(10) Measure the absorbance of the final reaction mixtures with a spectrophotometer at 562 nm (Ferene: 593 nm), using 1.000 cm

cuvettes and the reagent blank/precipitating-reducing solution (1:1) as blank.

(11) Prepare a calibration curve from the measured absorbances of the iron standard working solutions and read the unknown samples from the curve, or calculate the results by means of linear regression. Multiply the results by a factor 3 to account for the dilution of the original samples (see section 7.4, step 3).

(12) Analyze all iron standard working solutions and patient samples in duplicate.

The method can be adapted for use with smaller sample volumes, as long as the sample: precipitating-reducing-reagent: chromogen-reagent remains 2:2:1[26, 32]; semi-micro spectrophotometer cuvettes will be required. The method has been successfully automated.[28, 37, 61]

7.5 Quality Control

Three levels of quality control serum pools should be analyzed, in duplicate, with every standard curve and with every batch of specimens analyzed. Because lyophilization may result in significant amounts of easily split-off hemoglobin iron which is then also detected in the assay,[23] the use of frozen or liquid quality control materials is recommended. However, excellent precision has been obtained with freeze-dried materials. If use of lyophilized materials is contemplated, the laboratory must first verify that assigned values are recovered accurately and that acceptable precision is obtained for materials with, or materials without assigned values. Preparation of suitable quality control material is given in Appendix B.

Coefficients of variation somewhat higher than those for the serum iron concentration determination only, may be expected because of the additional sample preparation step (saturation with iron and removal of the excess iron; see Section 7.4). Control data, over several years, of in-house quality control preparations from one of the laboratories may be summarized as follows.

Table 3. TIBC Control Data over Several Years of In-house Control Preparations.

level	n	mean mg/L	S.D. mg/L	C.V. %
1¶	92	2.856	0.12	4.2
2¶	92	3.707	0.17	4.7
3¶	92	4.966	0.18	3.7
1¶¶	33	2.605	0.09	3.5
2¶¶	33	3.692	0.13	3.5
3¶¶	33	5.021	0.16	3.2

¶ = in-house preparation #1
¶¶ = in-house preparation #2

7.6 Interferences and Sources of Error

7.6.1 Contamination

A major source of error may be contamination of glassware and disposable materials (see Section 7.2.3).

7.6.2 Lot-to-Lot Variation of Magnesium Carbonate

A reproducible amount of magnesium carbonate must be added in the TIBC procedure. Because of lot-to-lot variation, the amount to be added must be reverified with each new lot of magnesium carbonate used by determining the TIBC of at least eight paired specimens at each of three levels (low, normal, high) in parallel, using the old and the new lot (also see NCCLS document EP9 *Method Comparison and Bias Estimation Using Patient Samples*). Adjust the quantity of magnesium carbonate until the average bias for the specimens is within +/- 5%.

7.6.3 Hemoglobin Iron

A second source of error is inclusion of (some of the) hemoglobin iron in the measurement of TIBC:

- grossly hemolyzed specimens are not acceptable.
- precautions should be taken to minimize hemolysis when drawing specimens. (Please see NCCLS document *H3 Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture*.)

- hemoglobin iron interference is minimized by the use of ascorbic acid as reducing agent.

7.6.4 Copper and Zinc

The colorimetric determination of iron is subject to interference by copper and zinc.[28, 38-42] Copper and zinc interference is significantly minimized by the inclusion of thiourea in the chromogen reagent[28] because these elements are avidly chelated by thiourea.

7.6.5 Control Materials

Lyophilized control materials and specimens are generally not suitable because the acid reagent may liberate iron from lyophilized hemoglobin in reconstituted serum.[21] If use of lyophilized materials is contemplated, the laboratory must first verify that assigned values are recovered accurately and that acceptable precision is obtained for materials with, or for materials without assigned values.

7.6.6 Plasma

When plasma samples are to be used for the determination of TIBC only, heparin must be used as anticoagulant (Section 4).

7.6.7 Diurnal Variation

In the interpretation of TIBC (and serum iron and % transferrin iron saturation) results, reports of marked diurnal variation must be taken into account.[43-48]

7.7 Reference Range

TIBC values for adult females and males generally do not differ significantly.

Values, as published, include:

females: 250 - 450 μg/dL (2.5 - 4.5 mg/L; 44.8-80.6 μmol/L) [53]
males: 306 - 429 μg/dL (3.06 - 4.29 mg/L; 54.8-76.8 μmol/L) [52]
females, males: 45 - 70 μmol/l [51]

TIBC values found in the National Health and Nutrition Examination Surveys, NHANES I (1971-74) and NHANES II (1976-80) were determined without the addition of thiourea to the chromogen reagent. The values, by age and sex, [54, 55] have been corrected for an estimated copper interference[24] in the method. A correction of 9.65% was determined by measuring 101 specimens, in parallel, with one chromogen reagent containing thiourea, and one without thiourea added.

Table 4. TIBC Values, NHANES I (1971-74) and NHANES II (1976-80)

		mean			percentile ranges					
age group (yrs)	n	mg/L	μg/dL	μmol/L	5th mg/L	95th mg/L	5th μg/dL	95th μg/dL	5th μmol/L	95th μmol/L
2-11	4995	3.44	344	61.6	2.61	4.28	261	428	46.7	76.6
12-17	2810	3.56	356	63.7	2.79	4.22	279	422	50.0	75.6
18-44	9850	3.31	331	59.3	2.66	3.96	266	396	47.6	70.9
45-74	9950	3.24	324	58.0	2.43	4.05	243	405	43.5	72.5

Preliminary data from NHANES III (1988-94) by age, gender, and race are given in Appendix A.

8 Calculation of Percent Transferrin Saturation

8.1 Transferrin

For transport purposes throughout the body iron in plasma is bound to transferrin, a beta-globulin. Although some investigators have also implicated alpha2-macroglobulin and the immunoglobulins as having a role in plasma iron binding and transport,[62, 63] there is general agreement that their role is quantitatively negligible.

Numerous genetically-controlled variants of human transferrin have been demonstrated by starch gel electrophoresis, but all appear to be similar in their ability to transport and deliver iron to the tissues.[64,65] Transferrins are composed of two metal binding lobes of approximately equal size; each lobe is ellipsoid-shaped and contains a metal binding site buried below the surface of the protein in a hydrophilic environment. In addition to Fe(III), transferrin will accommodate a number of other multivalent metal ions, e.g., Cu(II), Cr(III), Co(III), and Mn(III). Although probably not physiologically important, many of these metal ions have served as useful spectroscopic probes in the study of transferrin.

Transferrins contain 680 to 700 amino acids and two carbohydrate chains. The relative molecular mass has variously been reported as from 75 000 to 83 000,[66,67] and as 79 570 [68]; an average value of 80 000 appears to have been generally accepted. [58,69]

8.2 Transferrin Measurement

Transferrins may be assayed as a protein by immunological methods, e.g., immunonephelometric or immunoturbidimetric assay, radial immunodiffusion, and the concentration expressed as an amount of protein per liter, or they may be assayed by their capacity to bind iron.

Immunological methods should be referenced to the new international *Reference Preparation for Proteins in Human Serum, RPPHS-CRM 470*, with assigned values for 14 proteins.[70,71] Although radial immunodiffusion techniques have been used, they are not recommended because of excessive imprecision.[56]

Theoretically, 1 g of transferrin (average molecular mass 80 000) will bind 25 μmol (1.4 mg) of iron (relative atomic mass 55.85). Experimentally analyzing 567 patient samples, values ranging from 17.6 to 29.2 (total mean 22.3) have been reported for the ratio of serum iron-binding capacity (μmol/L) and transferrin (g/L).[58] The authors attributed this variability to imprecision inherent to the assays and not to any biological variability in the binding of iron to transferrin. For 29 pooled patient specimens covering a wide range of TIBC concentrations, the authors demonstrated a TIBC to transferrin ratio of 24.6, range 23.5 to 26.3, when precise and carefully standardized assays were used. Wide ranges of the ratio have been reported in other studies[63, 72-75]; these also appear to reflect a lack of standardization of the assays and imprecision, especially in the radial immunodiffusion assays used.

Other discrepancies in the agreement between TIBC and immunologically measured transferrin have been reported and were most likely caused by a lack of standardized calibration materials and variability in the removal of excess iron in the TIBC methods.[76] In a series of patient specimens, a range of about 10% in the ratio of TIBC to transferrin was found to be associated with changes in serum ferritin, unsaturated iron-binding capacity (UIBC), TIBC, and % transferrin saturation.[58] Unusually high TIBC results, compared to transferrin concentration, has been reported in patients with very high ferritin concentrations, with fulminant hepatitis, and in patients treated with desferrioxamine.[68]

8.3 % Transferrin Saturation

For most clinical applications, the percent transferrin saturation may be calculated as follows.

From the serum iron concentration and the directly measured total iron-binding capacity (see Section 7):

$$\text{transferrin saturation}\,(\%) = \frac{\text{serum iron concentration}\,(\text{mg/L})}{\text{TIBC}\,(\text{mg/L})} \times 100 \qquad (1)$$

From the serum iron concentration and the measured unsaturated iron-binding capacity

$$\text{transferrin saturation}\,(\%) = \frac{\text{serum iron concentration}\,(\text{mg/L})}{\text{serum iron concentration}\,(\text{mg/L}) + \text{UIBC}\,(\text{mg/L})} \times 100 \qquad (2)$$

From the serum iron concentration and the (immunologically) measured transferrin concentration, assuming the molecular mass of transferrin to be 80 000

$$\text{transferrin saturation}\,(\%) = \frac{\text{serum iron concentration}\,(\text{mg/L})}{\text{serum transferrin concentration}\,(\text{mg/L}) \times 1.4} \times 100 \qquad (3)$$

8.4 Reference Range

Transferrin is usually about one third saturated with iron. Published values include:

females, males: 33%- 35% [51, 52]
females, males: 20% to 50% [53]

Transferrin saturation values found in the National Health and Nutrition Examination Surveys, NHANES I (1971-74) and NHANES II (1976-80), by age groups, were as follows; no significant differences between males and females were found.[54,55]

Table 5. % Transferrin Saturation, NHANES I (1971-74) and NHANES II (1976-80)

age group (yrs)	n	mean %	5th %-ile	95th %-ile
2-11	4995	23	10	43
12-17	2800	25	14	55
18-44	9390	30	17	52
45-74	9950	30	15	52

Preliminary data from NHANES III (1988-94) by age, race, and sex are given in Appendix A.

8.5 Interpretation of Results

- Transferrin saturation of < 16% in adults is widely regarded as indicative of iron deficiency; it may, however, occur in chronic disease in the absence of iron deficiency.[77] There is substantial evidence of age-related changes in iron status indicators in infancy, childhood, and adolescence.[78-82] It has been proposed that transferrin saturation values of < 12% in 1-2 year olds, < 14% in 3-4 year olds, and < 15% in 5-10 year olds is indicative of iron deficient erythropoiesis.[24]

- The determination of % transferrin saturation has been recommended as a screening test for iron overload.[18, 83] If the % transferrin saturation is > 60% for males, or > 55% for females, the iron and TIBC measurements should be repeated and, if still found to be elevated, further diagnostic testing should be performed.

- Transferrin concentration and, thus, TIBC are relatively stable over time. Serum iron concentration, on the other hand, may vary over a 24-hour period by as much as 1.1 mg/L (112 μg/dL; 20 μmol/L).[43-48] Therefore, the % transferrin saturation can vary considerably without a change in iron stores.[84]

- Routine measurements of serum iron, TIBC, and % transferrin saturation, followed by determination of serum ferritin when the % transferrin saturation is abnormally low or high, appears to be the most cost-effective method of detecting patients with uncomplicated iron deficiency and with iron overload disease, *e.g.*, (idiopathic) hereditary hemochromatosis.[85, 86]

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Appendix A. (Semi)automation of the Measurement of Serum Iron and the Total Iron Binding Capacity

A1. Development

Serum iron and total iron binding capacity, TIBC, measured by modifications of the method described by Ramsay,[1] have been successfully adapted for use with Technicon auto analyzers.[2-6] With auto analyzers no longer available, the methods described in Sections 6 and 7 have been further modified at the CDC for the Third National Health and Nutrition Examination Survey (NHANES III), to be performed with a rapid flow analysis system (Astoria-Pacific International, Clackamas, OR).[7]

A2. Principle of the Method

Iron is released from transferrin by the addition of acid to the serum. The iron is reduced to Fe(II) and determined quantitatively by photometric measurement of the absorbance of the violet complex formed between Fe(II) and FerroZine reagent; the reagent also contains thiourea to complex Cu(II). For the determination of TIBC, saturation of transferrin with iron and removal of excess iron by magnesium carbonate has not been automated.

A3. Specimens

For a single determination of serum iron and TIBC a 1-mL serum specimen is preferable, but a sample volume of 0.5 mL can be analyzed.

Determination of serum iron requires a sample volume of about 0.3 mL, determination of TIBC a sample volume of 0.2 mL.

Also see Sections 4 and 6.7.2.

A4. Instrumentation, Chemicals and Materials

A4.1 Instrumentation

- vortex mixer;
- centrifuge;
- Astoria-Pacific International continuous flow analyzer (or equivalent) consisting of sampler (model 301), manifold (model 302), minipump (model 302), dialyzer with type "H" membranes and flow-rated tubing, controller (model 3104/314), photometer (model 305A/510), 15-mm flow cells, filter 562 nm, bandwidth 5nm;
- automatic pipets with 1 mL dispensing and sampling pumps or 2 mL dispensing and sampling syringes (e.g., Micromedix model 25000 or model Digiflex automatic).
- Data system, e.g., 286 computer, printer, chart recorder, and manufacturer- recommended software.

A4.2 Materials

- 0.25-mL disposable sample cups, conical bottom;
- 12- x 75-mm glass culture tubes, disposable, lot-tested for iron contamination;
- filtering columns, disposable;
- membrane filters, 0.45 μm.

Appendix A (Continued).

A4.3 Chemicals

In addition to the chemicals listed in Sections 6.2.2 and 7.2.2, the following are required:

- Brij-35,® 30% solution (23 lauryl ether; trade name ICI Americas, Inc., Wilmington, DE)
- Kemwash detergent solution (Astoria-Pacific International, Clackamas, OR)
- Sodium chloride

A5. Reagents

Compare to Sections 6.3 and 7.3: Reagents

- hydrochloric acid, 0.2 mol/L, with sodium chloride, 30g/L; prepare as needed
- NaCl-HCl-Brij solution. For each 150 samples to be analyzed add 20 drops, 30% Brij solution to 200 mL of reagent (1); prepare fresh daily
- HCl-NaCl-ascorbic acid solution. For each 150 samples to be analyzed add 1.0 g ascorbic acid and 10 drops, 30% Brij solution to 100 mL of reagent (1); prepare fresh daily
- acetate buffer, 0.75 mol/L; prepare fresh weekly
- FerroZine™, 0.7 g/L, with 1% (w/v) thiourea, in acetate buffer, 0.75 mol/L (see (4)); prepare weekly and filter (0.45 μm membrane filter)
- Brij-35 wash solution, 0.5 mL (30%)/L; prepare weekly
- hydrochloric acid, 0.1 mol/L; for preparation of iron standard solutions
- iron saturating solution, 4 mg/L

A6. Iron Standards Solutions

Compare to Section 6.4

For iron standard stock solution and iron standard intermediate solution, see Section 6.4.

From the iron standard intermediate solution, 50.0 mg/L, prepare working standards 300, 500, 800 μg/L Fe, and 1.0, 1.5, 2.0, 2.5, 3.0 mg/L Fe in HCl, 0.1 mol/L.

A7. Quality Control Materials

Three levels of quality control preparations should be analyzed in duplicate with every standard curve and with every 20 specimens analyzed. Quality control materials may be purchased commercially or prepared in-house (compare Appendix B). Pools are filter-sterilized, dispensed into glass vials, capped, and stored at -70 °C for maximum stability.

A8. Calibration Verification

Accuracy and precision of iron measurements may be verified with National Institute of Standards and Technology, NIST, SRM 1598, Inorganic Constituents in Bovine Serum, a frozen material with target values for serum iron, or with NIST iron standard SRM 937, iron metal, in HCl.

In each case at least three replicates of each dilution should be run as unknowns, against the working standards. A linear regression generated from the working standards as X and SRM 937 as Y should give a correlation coefficient of at least 0.98 with a slope of 1.00 +/- 0.05 and a Y-intercept of 0.0 +/- 1.0 μg/dL.

Appendix A (Continued).

A9. Determination of Serum Iron Concentration

Instrument set-up and instrument parameters are shown in Figure A1.

(1) Mix serum specimens well and filter about 0.3 mL serum with a disposable plastic filtration column into a 0.5 mL sample cup.

(2) Follow standard protocol as given in manufacturer's operations manual.

(3) Approximately 100 μL serum is required for each analysis. Analysis rate is 72 samples per h. Calibrate with ascending- and descending-order standard curves analyzed with every 60-80 specimens. Include quality control materials with every standard curve and every 20 samples.
(4) Change pump tubing and "H" membranes every two days.

(5) Use a strip-chart recorder to monitor for unusual changes in peak height or shape.

A10. Determination of TIBC

(1) With an automated pipet, dilute 0.2 mL well-mixed serum with 0.4 mL iron saturating solution, 4.0 mg/L (see Section A5, Bullet #(8) in 12-x75-mm tubes.

(2) Mix well with vortex mixer and allow to stand for at least 30 min.

(3) Add 100 mg basic magnesium carbonate to each tube of diluted serum. Mix well and allow tubes to stand for 45 min, re-mixing at 15 min intervals.

(4) Centrifuge the tubes at 2 500 x *g* for 10 min and decant the supernatant into 0.25-mL sample cups.

(5) Perform iron analyses according to Section A9.3

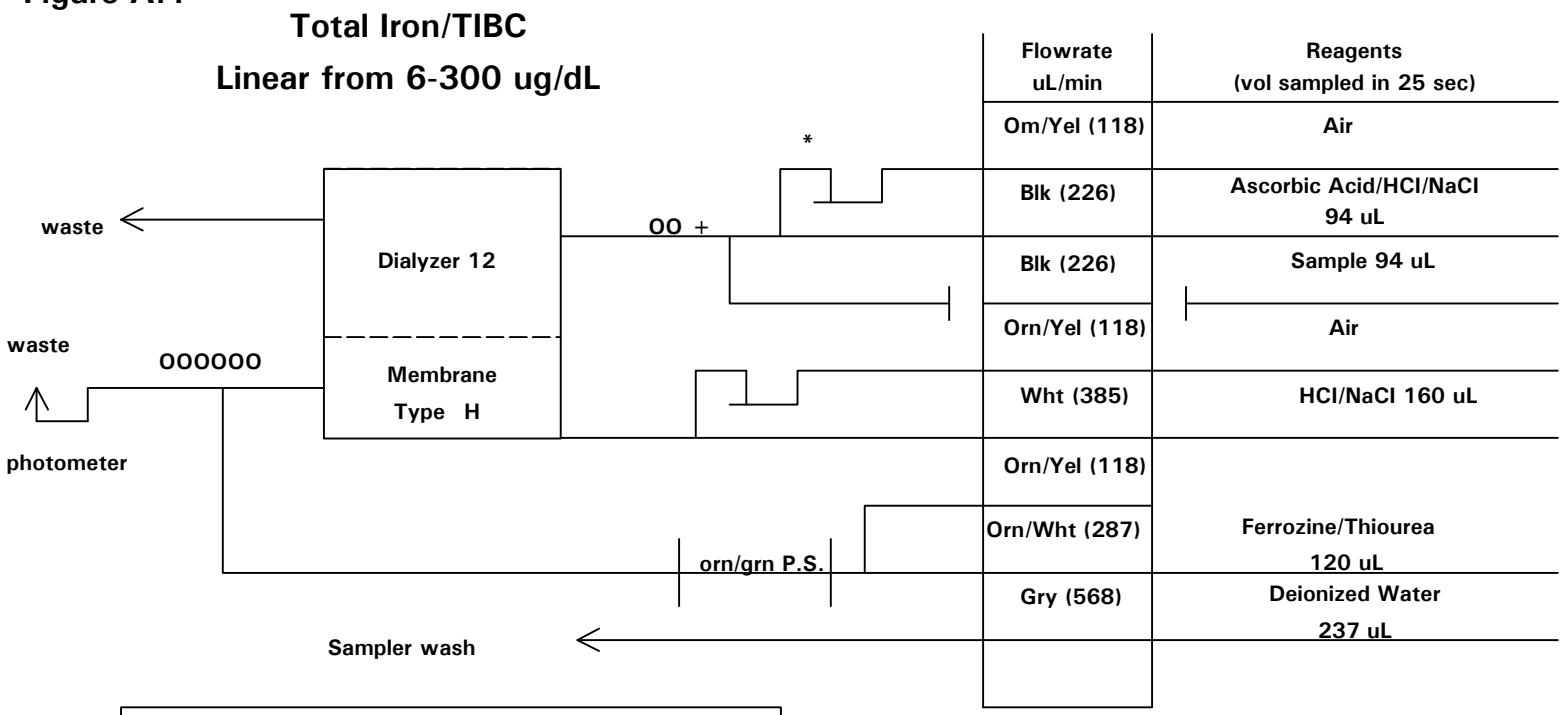
A11. Corrective Action

If the system is found to be "out-of-control," the following remedial actions are recommended:

(1) check the system for fibrin clots in samples or in metal connectors;
(2) replace "H" membrane;
(3) replace pump tubing;
(4) check the timing and the bubble gating.

Appendix A (Continued).

Figure A.1



Photometer Parameters	
Filter	570 nm
Flowcell	15 mm, stainless steel endcapped
Absorbance range	O.1 (AUFS)
Damping (RC)	2 sec
Sampler Parameters	
Analysis rate	72/hr
Sample time	25 sec
Wash time	25 sec
Pecking	off
Probe	Stainless steel

* Air solenoid valve
+ Coil, 0 = 5 turns
0.015 ID Polythylene
Ferrozine/Thlourea is delivered with 2 lines to get the proper volume at 287 uL/min flowrate

Manifold startup solution	Deionized water with Brij-35 (1 mL/L)
Cleaning solution	KemWash (ALPKEM P/N A01-5555-26) or 1 N NaOH

Appendix A (Continued).

A12. NHANES III, 1988-94; Serum Iron, Total Iron-Binding Capacity, and % Transferrin Iron Saturation

The (semi)automated methods have been used to determine the serum iron concentration, the TIBC, and the % transferrin saturation during the National Health and Nutrition Examination Survey III. Preliminary analysis of the data by age, race, and sex are shown in Figures A2, A3, and A4.

In summary, the serum iron mean concentration data show a gradual increase up to age group 20-30-years, a gradual decrease after age 40-years. There is no significant difference between non-Hispanic white and Mexican-American males; non-Hispanic black males test approximately 10% lower. There is no significant difference between non-Hispanic white and Mexican-American females; non-Hispanic black females test 15-20% lower. Non-Hispanic white females test about 10% lower than non-Hispanic white males, non-Hispanic black and Mexican-American females test 15-20% lower than their male counterparts.

With respect to TIBC, there are no apparent significant differences between males and females. Males show a gradual decrease in TIBC after age 15, females after age 30. Older non-Hispanic black females (after age 60) appear to test about 10% lower than their non-Hispanic white and Mexican-American counterparts.

The % transferrin saturation gradually increases to age 30 in males, to age 10 in females. Females of all age groups test about 5% lower than males, with % saturation in males 25-30%, in females 20-25%.

Appendix A (Continued).

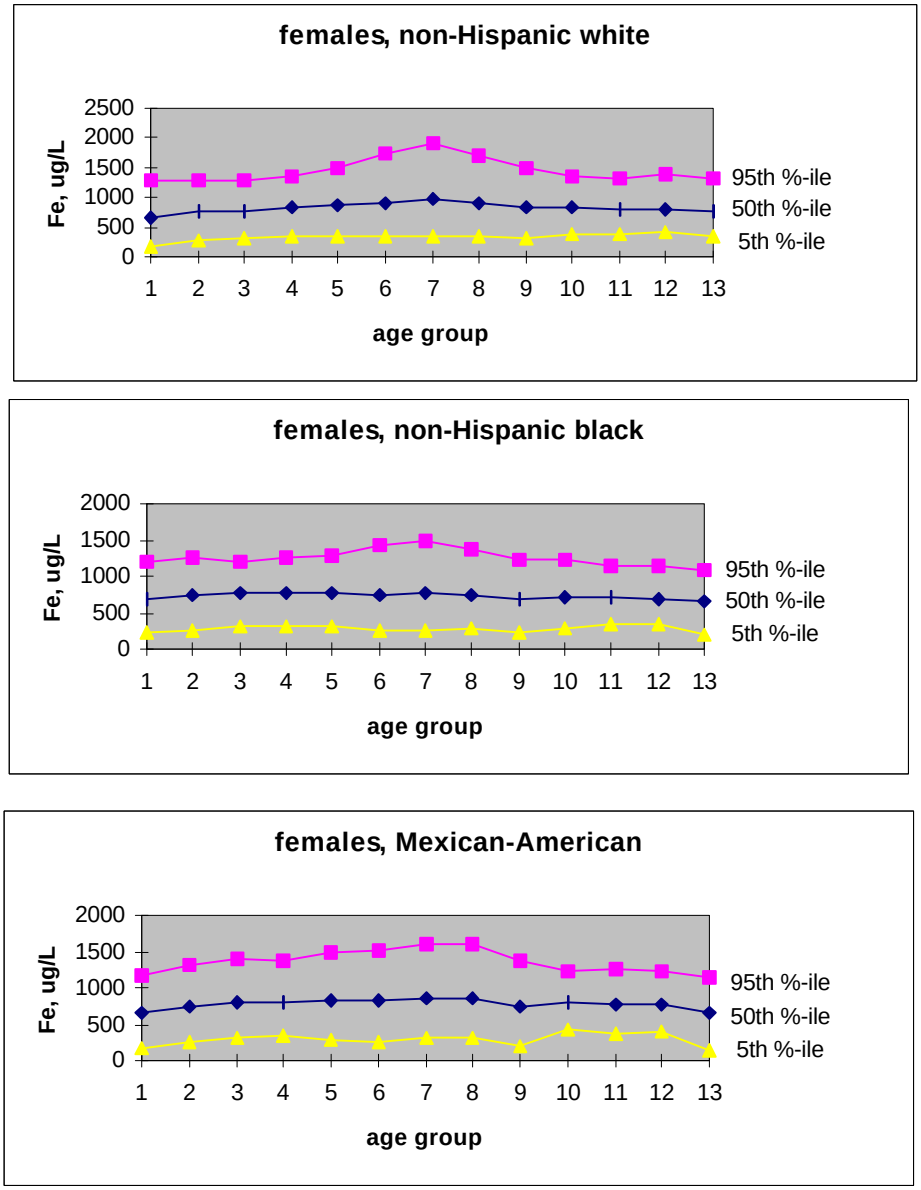
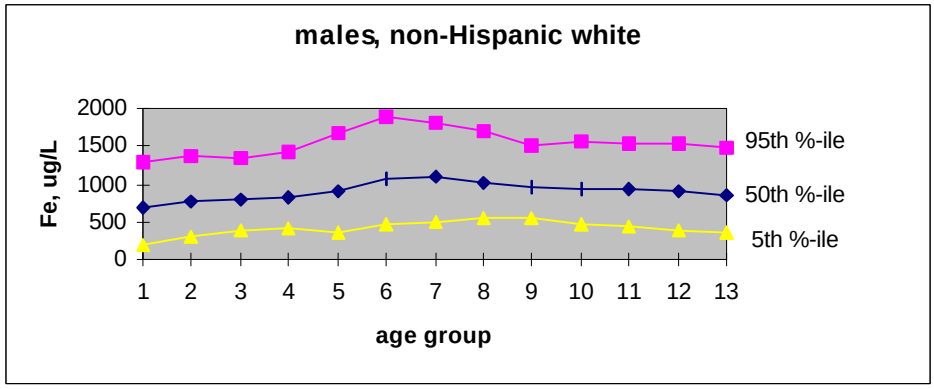


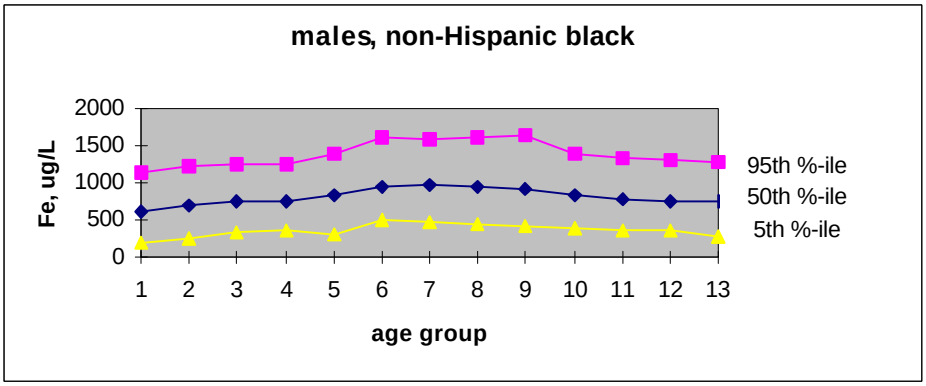
Figure A2(a). NHANES III (1988-94), serum iron, μg/L (preliminary data)

X axis

age group	age (yrs)	age group	age (yrs)
1	1 - 2	8	30 - 39
2	3 - 5	9	40 - 49
3	6 - 8	10	50 - 59
4	9 - 11	11	60 - 69
5	12 -14	12	70 - 79
6	15 -19	13	80 +
7	20 -29		

Appendix A (Continued).





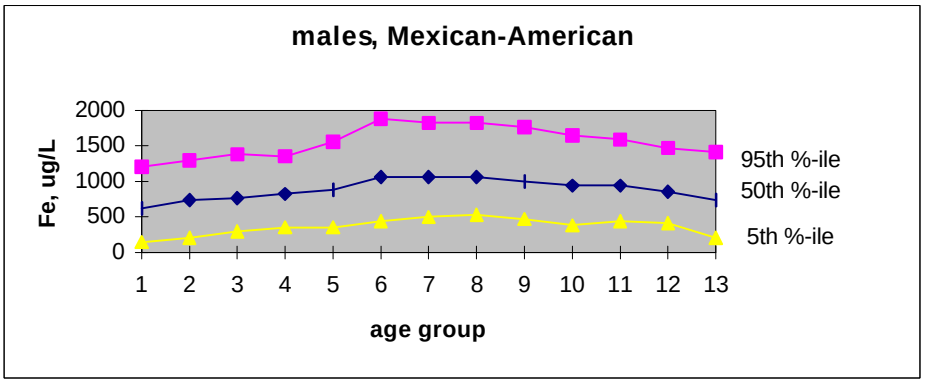


Figure A2(b). NHANES III (1988-94), serum iron, μg/L (preliminary data)

X axis

age group	age (yrs)	age group	age (yrs)
1	1 - 2	8	30 - 39
2	3 - 5	9	40 - 49
3	6 - 8	10	50 - 59
4	9 - 11	11	60 - 69
5	12 -14	12	70 - 79
6	15 -19	13	80 +
7	20 -29		

Appendix A (Continued).

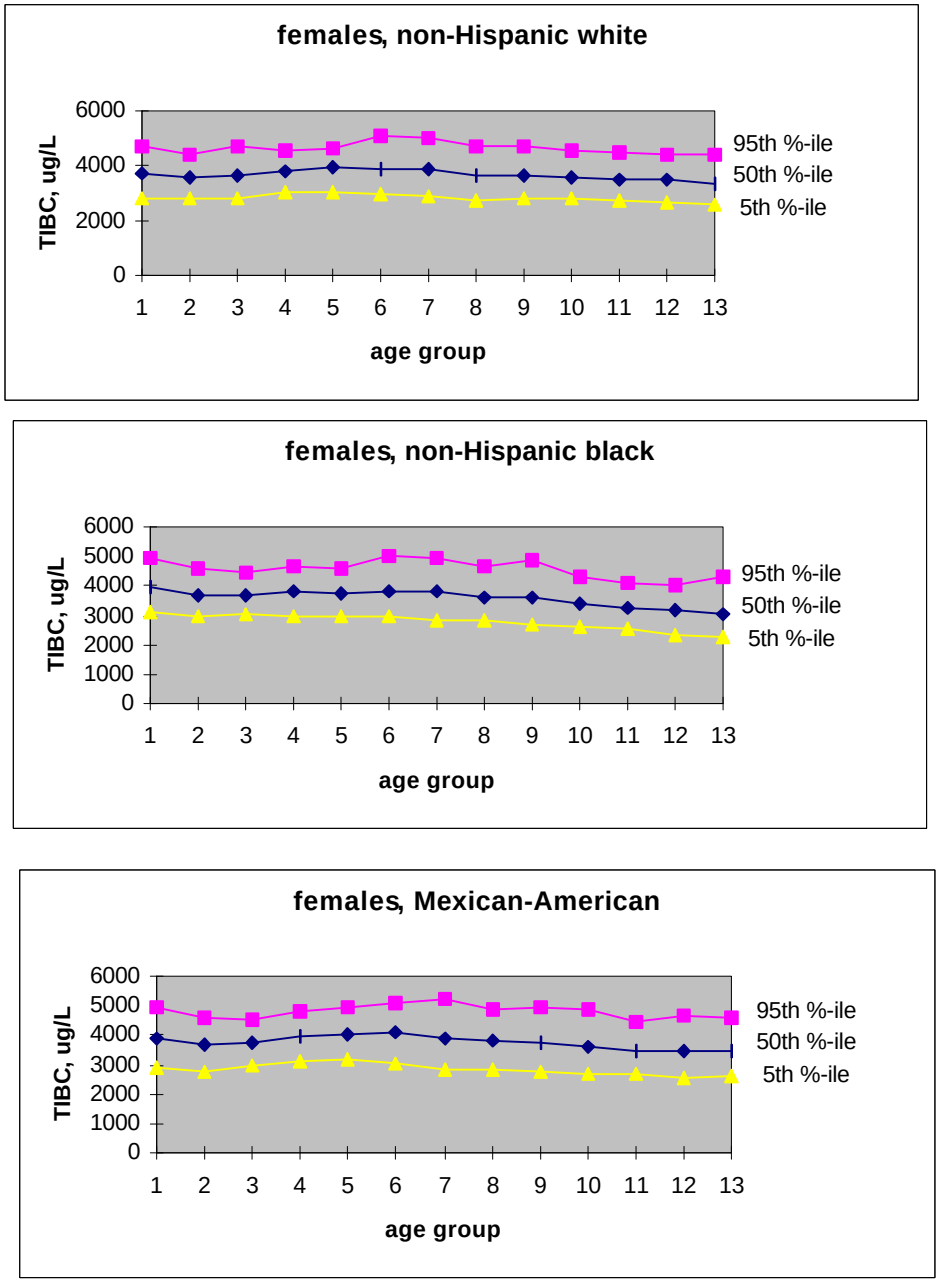


Figure A3(a). NHANES III (1988-94), total iron-binding capacity, μg/L (preliminary data)

X axis

age group	age (yrs)	age group	age (yrs)
1	1 - 2	8	30 - 39
2	3 - 5	9	40 - 49
3	6 - 8	10	50 - 59
4	9 - 11	11	60 - 69
5	12 -14	12	70 - 79
6	15 -19	13	80 +
7	20 -29		

Appendix A (Continued).

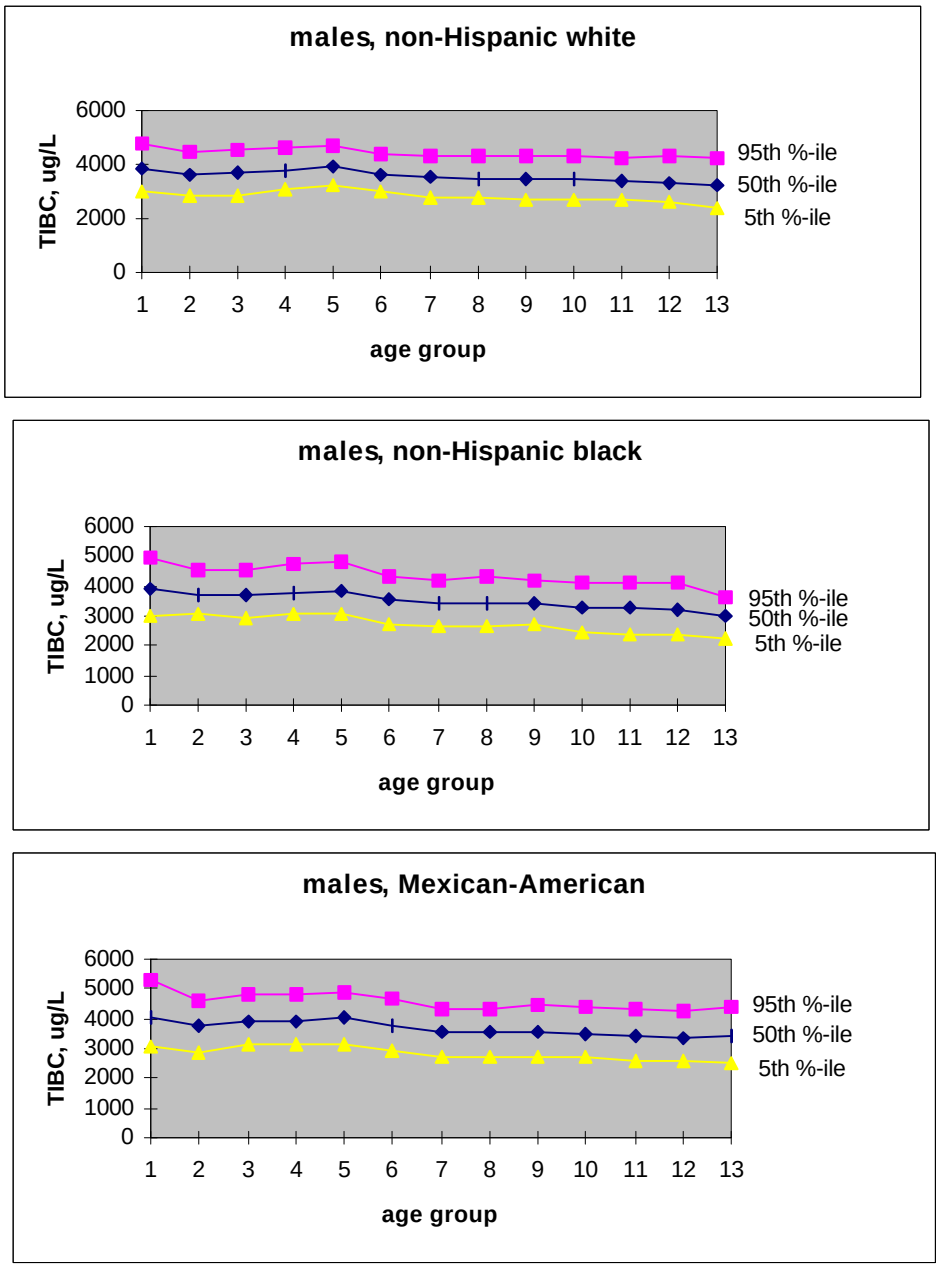


Figure A3(b). NHANES III (1988-94), total iron-binding, capacity, TIBC, μg/L (preliminary data)

X axis

Age group	age (yrs)	age group	age (yrs)
1	1 - 2	8	30 - 39
2	3 - 5	9	40 - 49
3	6 - 8	10	50 - 59
4	9 - 11	11	60 - 69
5	12 -14	12	70 - 79
6	15 -19	13	80 +
7	20 -29		

Appendix A (Continued).

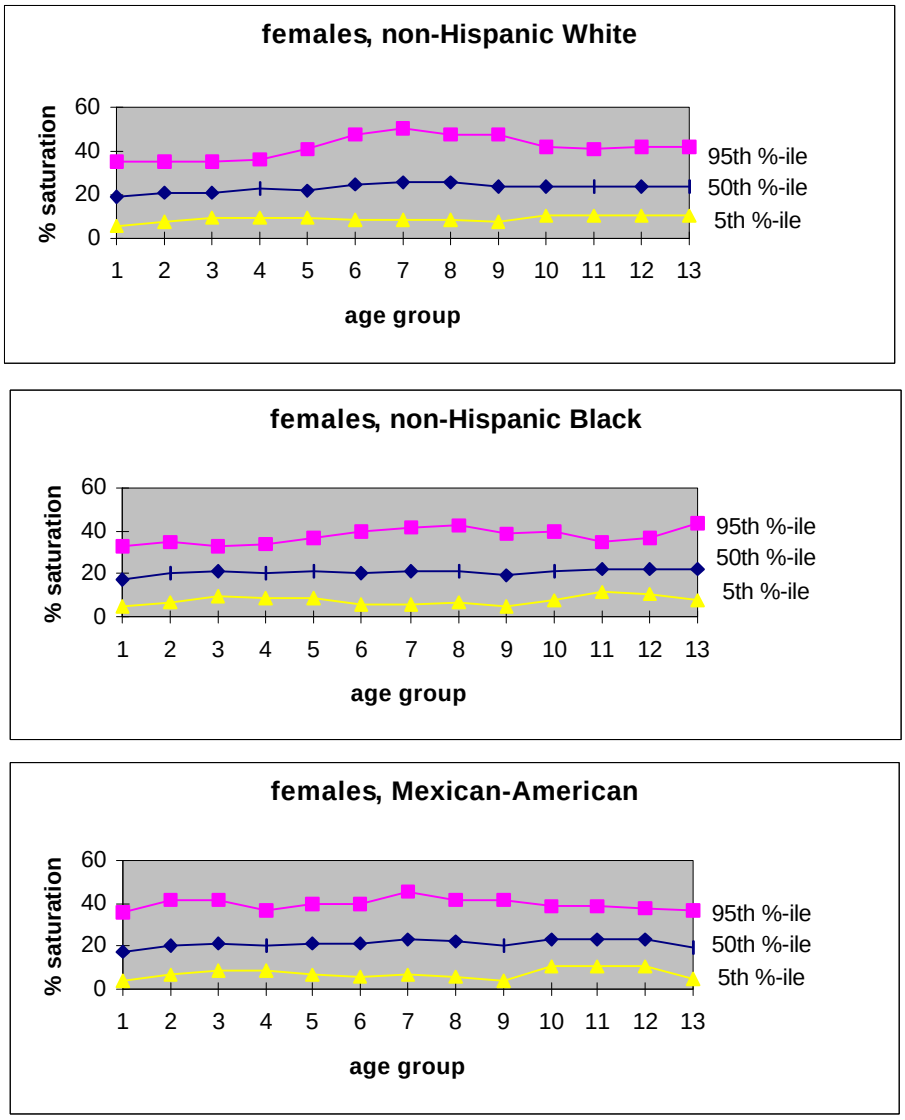


Figure A4(a). NHANES III (1988-94), percent transferrin saturation (preliminary data)

X axis

age group	age (yrs)	age group	age (yrs)
1	1 - 2	8	30 - 39
2	3 - 5	9	40 - 49
3	6 - 8	10	50 - 59
4	9 - 11	11	60 - 69
5	12 -14	12	70 - 79
6	15 -19	13	80 +
7	20 -29		

Appendix A (Continued).

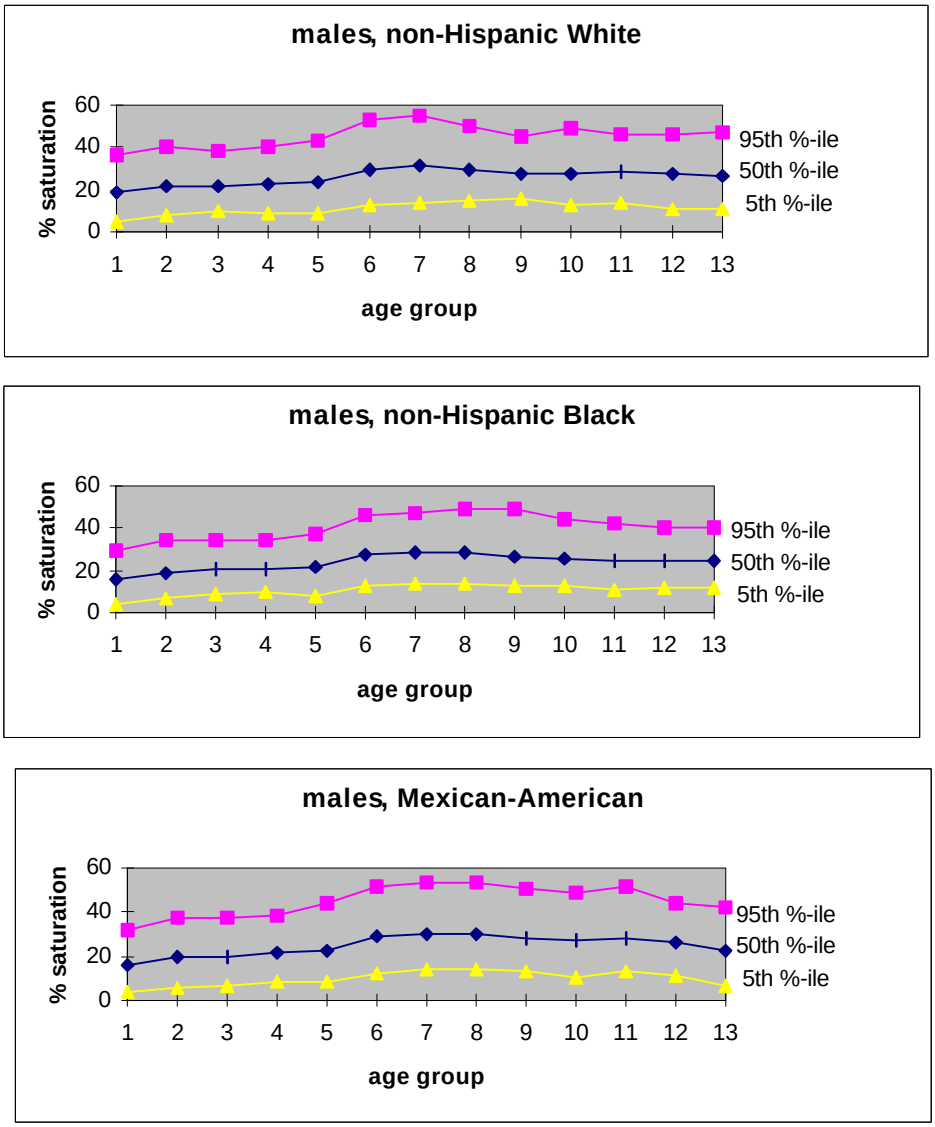


Figure A4(b). NHANES III (1988-94), percent transferrin saturation (preliminary data)

X axis

age group	age (yrs)	age group	age (yrs)
1	1 - 2	8	30 - 39
2	3 - 5	9	40 - 49
3	6 - 8	10	50 - 59
4	9 - 11	11	60 - 69
5	12 -14	12	70 - 79
6	15 -19	13	80 +
7	20 - 29		

Appendix A (Continued).

References to Appendix A.

1. Ramsay WNM. The determination of the total iron-binding capacity of serum. *Clin. Chim. Acta*.1957(2):221-226.

2. Giovaniello TJ, Bendetto G, Palmer DW, Peters T. Fully and semiautomated methods for the determination of serum iron and total iron-binding capacity. *J. Lab. Clin. Med*. 1968:71,874.

3. Huenteler JLA, van der Slik W, Persijn JP. Automated determination of serum iron and latent iron-binding capacity by continuous flow analysis without dialysis. *Clin. Chim. Acta.* 1972:37, 391-397.

4. National Center for Health Statistics, HANES I Hematology and Clinical Chemistry Procedures Developed or Utilized by the Center for Disease Control, Bureau of Laboratories, 1971-1975. Washington, D.C.: U.S. Government Printing Office; 1979:34-35.

5. Gunter EW, Turner WE, Neese JW, Bayse DD, Laboratory Procedures Used by the Clinical Chemistry Division, Centers for Disease Control, for the Second Health and Nutrition Examination Survey (HANES II) 1976-1980. Atlanta, GA: Centers for Disease Control; 1981: 12-15.

6. Gunter EW, Miller DT. Laboratory Methods Used by the Division of Environmental Health Laboratory Sciences, Center for Environmental Health, Centers for Disease Control for the Hispanic Health and Nutrition Examination Survey (HHANES) 1982-84. Atlanta, GA: Centers for Disease Control; 1986:13-16.

7. Gunter EW, Lewis BL, Koncikowski SM. Laboratory Methods Used for the Third National Health and Nutrition Examination Survey (NHANES III), 1988-94. Hyatsville, MD: Centers for Disease Control and Prevention; 1997: Sections VII-B, VII-E.

Appendix B. Preparation of Quality Control Material

Quality control preparations must be included with each run of serum iron/total iron binding capacity determinations. Suitable quality control material at three concentration levels may be prepared as follows.

Perform all steps of drawing, handling, and processing of the blood and serum using sterile techniques.

(1) Collect blood from apparently healthy normal subjects, using a thin-walled 16-gauge needle, into iron-free polyethylene plastic bottles.

(2) Allow blood to stand at ambient (room, 18-25 °C) temperature for about 16 hours.

(3) "Ring" the clot carefully and centrifuge at about 5 000 X g for 20 minutes.

(4) Decant the serum carefully, taking care to avoid contamination with red cells.

(5) Recentrifuge the serum at 5 000 X g for 20 min and again decant the serum carefully.

(6) Pool the serum from the donors and divide the pool into three lots.

(7) Add 1 mL stock iron standard solution, 1.000 g/L (see Section 6.4.1), i.e., 1 mg Fe,to each liter of serum of one of the lots obtained in step 6; add 1 mL iron-free water to each liter of serum of a second lot obtained in step 6; mix thoroughly. A third level of control material can be prepared by filtration of the third lot obtained in step 6 on a hollow-fiber column which removes about 30% of the water content of the serum.

(8) Aliquot the materials obtained in step 7 into iron-free vials and store at < -35 °C.[23]

Serum iron and iron binding capacity values are assigned to the three lots of quality control material by repeated analyses.

The quality control preparations should not be lyophilized. Lyophilized material has been identified as a source of inter-laboratory variation. [21, 53]

References to Appendix B.

1. International Committee for Standardization in Haematology. The measurement of total and unsaturated iron-binding capacity of serum. *Br. J. Haematol.* 1978;38:281-290.

2. Fairbanks VF, Klee GG, Biochemical aspects of hematology. In: Tietz NW, ed. *Textbook of Clinical Chemistry.* Philadelphia: WB Saunders Co.;1986:1582.

Summary of Comments and Working Group Responses

H17-P: *Determination of Serum Iron and Total Iron-Binding Capacity; Proposed Standard*

Message to the User

1. Message to the user, page i, states "The method is useable and linear over a wide range of iron concentrations, interference is negligible, and precision is adequate." I found absolutely no data in this document to justify that linearity, interference, or precision were examined, much less that these features deserve a favorable evaluation.

- **A major source of interference is the presence of copper; the addition of thiourea to the chromogen reagent minimized this. (See also Reference 29.) For linearity and precision see Comment 5.**

2. In the "Message to the User" it is implied that this is a reference method, but in paragraph 2.0 it seems to be recommended for routine testing. What is the committee proposing? Why is this method superior to available methods for iron and UIBC that do not require centrifugation?

- **This method is recommended for the accurate determination of serum iron. However, individual laboratories may decrease the required sample size and/or automate the method(s) for routine use. When comparing different methods in routine use, large discrepancies have been reported (see References 18, 30, and 31). The method as described is superior because it minimizes interference by hemoglobin iron, copper, and zinc, due to its precision (see Sections 6.6.3, 6.8, and 7.5.2), and its linearity. (See the response to Comment 5.)**

  Centrifugation of the sample/reagent mixture ensures the removal of precipitates and the absence of turbidity in the final reaction mixture.

Foreword

3. In the Foreword (page ix) the comment is made that serum ferritin is relatively sensitive and specific in uncomplicated iron deficiency. I cannot support that statement, based on my reading of the literature, as well as my experience at MetPath where we perform up to 10,000 iron and TIBC assays a day with about 25 percent of these reflexing to a serum ferritin. A very low serum ferritin (less than 10 mcg/L) is highly specific for low iron stores, but such a low level has a sensitivity of only around 50 percent. The sensitivity and specificity of the ferritin assay are highly dependent on the cutoff used. A cutoff of 50 mcg/L, for example, has a sensitivity of around 90 percent but a specificity that is abysmal.

- **The working group agrees and has reworded the appropriate passages.**

Introduction

4. The Introduction section incorrectly and inconsistently states that TIBC is relatively insensitive as a test for iron deficiency. That is true for serum iron and may be true for percent saturation, but is not true for TIBC. The Introduction further states that serum ferritin is more sensitive. That is not true. A very low serum ferritin is highly specific, not highly sensitive. When you make serum ferritin highly sensitive, the specificity is poor.

- **The working group agrees and has reworded the appropriate passages (see also Comment 3).**

Principle of the Methods

5. How accurate is the method, what is the expected precision, and what is the linear range?

- **There is no definitive method to which (candidate) reference methods can be compared. The average recovery of Fe-spiked specimens covering the normal adult serum iron range was 104.2% (see Reference 29) and the ratio of TIBC to directly measured transferrin concentration is very close to the theoretically expected value. (See Reference 60.)**

  The coefficient of variation at 600-700 μg Fe/L (60-70 μg/dL; 10.7-12.5 μmol/L) is less than 4%, at 1.5 mg Fe/L (150 μg/dL; 26.9 μmol/L) around 2%, and at 3.0 mg Fe/L (300 μg/dL, 53.7 μmol/L) around 1%.(See Section 6.6.2.)

  There is no interference from Cu(II) and Zn(II).

  A linear range up to 4 mg/L Fe has been reported for FerroZine™ as chromogen (see reference 43); up to15 mg/L for ferene as chromogen (see reference 52). With FerroZine™ as chromogen linearity of the method up to 3.0 mg/L (300 μg/dL; 54 μmol/L) has been reported (See Reference 30), and with ferene as chromogen up to 7 mg/L (700 μg/dL, 125.7 μmol/L). (See Reference 29.)

6. What has been the result of interlaboratory studies using these methods?

- **Data from multiple laboratories analyzing the same specimen(s) are not available at this time.**

Method for Determination of TIBC, Section 9.0

7. The TIBC method requires the addition of "0.30 g" of basic magnesium carbonate to each sample. How should this be accomplished?

- **The required amount of basic magnesium carbonate has been adjusted to 300 +/- 50mg. This amount can be weighed and added, or as experience in the NHANES laboratories at the Centers for Diseases Control and Prevention has shown, the laboratory can make a scoop from, for example, an appropriate sample cup to dispense, with practice, a reproducible amount.**

8. How does one determine the correct amount of magnesium carbonate when changing lots? when setting up the test in a new lab?

- **The TIBC of at least eight paired specimens at each of three levels (low, normal, high) is determined in parallel, using the old and the new lot (also see NCCLS document EP9 *Method Comparison and Bias Estimation Using Patient Samples*). Adjust the quantity of magnesium carbonate until the average bias for the specimens is within +/- 5%.**

Quality Control, Section 10

9. The usefulness of the method is very limited since one cannot use lyophilized control materials. What happens with proficiency survey materials, which are usually lyophilized?

- **Because the acid reagent may liberate iron from lyophilized hemoglobin in reconstituted serum, lyophilized control/proficiency assessment materials remain a problem (compare References 19, 61, 62). Good results, however, are obtained with commercial liquid or frozen control materials. It should be recommended that proficiency assessment programs include such materials for the evaluation of serum iron and iron-binding capacity.**

10. The recommended quality control material would provide only one level for total Iron-Binding Capacity. The preparation would be tedious, involving aliquoting and freezing the serum pads.

I appreciate the concerns about lyophilized material, but would suggest that liquid controls be considered.

- **See response to Comment 9. A third level of control material for the iron-binding capacity may be prepared by hollow-fiber column filtration of part of the batch of control material; this removes about 30% of the water content of the serum. (See also Appendix A12.)**

Interferences and Sources of Error, Section 12.0

11. A very small amount of iron can come from hemoglobin which escapes by hemolysis; this problem easily happens during the routine laboratory jobs. Such hemolysis is usually not so visible that laboratory workers kick out the specimen as is not suitable for the determination of serum iron concentration. You indicate the limit of the interference of the method on page 9, 12.0 in the document; however, it is hard for us to find the acceptable specimen only guided as "visibly hemolyzed specimens are unacceptable." Please clearly state how much we estimate erroneously higher in serum iron concentrations at a certain hemoglobin concentration in the specimen.

- **Serum specimens are usually not perceived as hemolyzed until the serum hemoglobin concentration exceeds 300 mg/L (0.02 mmol/L). However, hemoglobin interference has been minimized by the use of ascorbic acid as the reducing agent and it has been found that plasma hemoglobin levels up to 800 mg/L (80 mg/dL; 0.05 mmol/L) did not significantly alter the results of serum Fe determinations (V.F. Fairbanks, Mayo Clinic: unpublished results; personal communication).**

References

12. Of 52 references cited, 36 date prior to 1980, 47 date prior to 1985. Without doing a literature search of my own, I can hardly believe that topics addressed in this document have received so little recent attention in the literature.

- **Serum iron has interested researchers since the publication of Das Serumeisen (Heilmeyer and Plotner) in 1937, and much work on methods to determine serum iron and iron-binding capacity was done in the nineteen-fifties and nineteen-sixties. The working group believes it is useful to cite the original literature, and the more recent literature only as it is relevant to the methodologies discussed in this document.**

Summary of Delegate Voting Comments and Responses

H17-A: *Determination of Serum Iron-Binding Capacity and Percent Transferrin Saturation; Approved Standard*

General

1. It seems that the heart of this document is the actual method for the determination of serum iron. Given what the abstract says (linear over a wide range of iron concentrations, no interference, adequate precision) about the attributes of the method, not to mention other features (tedious, requires large sample size and meticulous processing), it seems that this is actually a reference method. It does not seem appropriate for NCCLS to disguise a reference method by calling it a "recommended method" (see title to Section 6).

- **NRSCL8-A defines the term Reference Method as: A thoroughly investigated method, in which exact and clear descriptions of the necessary conditions and procedures are given for the accurate determination of one or more property values, and in which documented accuracy and precision of the method are commensurate with the method's use for assessing the accuracy of other methods for measuring the same property values, or for assigning reference method values to reference materials. The working group agreed to use the alternative term Recommended Method to avoid confusion.**

Foreword

2. The Foreword is a comprehensive presentation of the physiology of iron related to specific medical conditions —in fact, it reads quite well. It does not, however, suggest anything that directly links this information to the method to which it serves as a foreword.

- **The format of the Foreword was chosen to put the measurement of iron status in perspective. The Foreword presents the physiology of iron, relates serum iron, transferrin, and ferritins to iron deficiency and iron overload, and, in the final two paragraphs, leads, to the body of the document, i.e., the determination of serum iron, the determination of iron-binding capacity, the determination of % transferrin saturation.**

Section 6.7

3. The light sensitivity of ferene should be mentioned, and the reagent should be stored protected from light.

- **There is no mention of light sensitivity in the original reference (Reference 52).**

Sections 6.6 and 7.5

4. Under section 6.6 and 7.5, referring to quality control for serum iron and TIBC assays, the standard recommends against using lyophilized quality control material because lyophilization splits off hemoglobin iron, which is detected by the serum iron and TIBC assays, increasing the results. This recommendation and the reasoning behind it are missing some facts about the assayed values claimed for lyophilized control products.

   Assayed values for quality control material are determined after the lyophilization process and upon reconstitution and, therefore, the package insert values already reflect any split off hemoglobin iron. Manufacturers then assign control stability based on recovering those values over the product shelf life. Freeze-drying cycles used by commercial manufacturers are fine-tuned so that each analyte claimed on a package insert will hold its recovery over the product shelf life. This is the benefit of using products with validated claims for stability.

The same facts hold true for unassayed controls that claim these analytes in their package inserts as meeting the product shelf life. However, as is always done in good laboratory practice, laboratories should then set their own means and ranges for their method and laboratory.

Based on these facts, the recommendation against laboratories using lyophilized controls would be misleading to laboratories and should be removed.

- **The commentor agrees there is danger of split-off hemoglobin iron and then states that assay values in their facility are determined after lyophilization and reconstitution. There is, however, no indication that all manufacturers follow such a protocol. The general warning Lyophilized specimens and control materials are generally not suitable . (See Section 6.7.4) thus appears to be valid. However, the following sentence has been added to Section 6.7.4: They are, however, suitable if values are assigned after rehydration of the lyophilized product.**

Section 7.3.1

5. In Section 7.3.1, the iron saturating solution used for TIBC measurements contains 2 mg/L iron. One part of serum is combined with two parts of this saturating solution. The maximum UIBC that can be measured in this manner is 4 mg/L. In iron deficiency anemia, the UIBC can be higher. The original paper by Ramsay (*Clin Chim Acta* 2:221-226, 1957) describing this TIBC method used a ferric chloride solution containing 5 mg/L of iron and 100 mg of magnesium carbonate per 1mL of saturating solution. I would recommend using a minimum concentration of 3 mg/L ferric chloride for the Candidate Standard method for TIBC. The 300 mg quantity of magnesium carbonate specified in the Candidate Standard should be more than adequate for this increase.

- **Reviewing the NHANES III (1988-94) serum iron/TIBC raw data (by age, race, gender; 12,900 females, 11,800 males), occasional very low serum iron (<100 Fg/L) or very high TIBC (>6,500 Fg/L) individual values were identified. An iron saturating solution 2 mg/L could, in such cases, contain insufficient iron to fully saturate all available transferrin. The working group does not have data/experience with saturating solutions 3 mg/L, but does have extensive data/experience with saturating solutions 4 mg/L. Therefore Section 7.3.1 has been changed to read:**

  7.3.1 Iron Saturating Solution, 2 mg/L; Alternatively 4 mg/L

  Dilute 1.0 mL (alternatively 2.0 mL) stock iron solution,

  And, the following sentence has been added to Section 7.4 (3): Specimens with low serum iron concentration, i.e., <200 μg/L (20 μg/dL; 3.6 μmol/L) may require 2.0 mL of iron saturating solution, 4 mg/L (Section 7.3.1).

Section 7.7

6. In Section 7.7, the TIBC reference range values appear to be discrepant: the upper reference range limit in umol/L is given as 80.6 for females, 76.8 for males, but only 70 for "males, females."

- **Section 7.7 states Values as Published which indicates there may be/are discrepancies. These values also cite their references.**

Section 8.4

7. In Section 8.4, the reference interval of 33-35% for % transferrin saturation for females is incorrect. We do not have access to references 53 or 54. Other references give values of 20-

45%. References 53 and 54 should be reviewed and the correct reference interval provided.

- **Most hematology texts indicate that transferrin is about one third saturated with iron, published values, however, vary significantly. Section 8.4 gives an example of this. However, an editorial error has occurred in Section 8.4 and has been corrected to indicate both females and males for both reference ranges indicated.**

  females, males: 33%-35% [53, 54]
  females, males: 20%-50%[55]

  The most extensive data regarding the reference range is undoubtedly the NHANES I and II data in Table 5, and the NHANES III data, Appendix A, Figure A4(a) and A4(b).

Appendix A

8. Appendix A on (semi)automation recommends outmoded and outdated technology when more modern suitable alternatives are available.

- **The commentor may have misunderstood the aim of Appendix A. This appendix describes how the methods specified in Sections 6 and 7 can be/have been (semi)automated. Appendix A does not recommend this as *the* automated method. Certainly, there is more up-to-date equipment. Regrettably this equipment and the methods developed for this equipment are the cause of significant variability of the iron determinations (see References 30 and 31).**

Related NCCLS Publications**

C42-A **Erythrocyte Protoporphyrin Testing; Approved Guideline (1996).** This document contains recommended guidelines for the measurement, reporting, and interpretation of erythrocyte protoporhyrin using hematofluorometric and extraction measurement methods.

EP5-T2 **Precision Performance of Clinical Chemistry Devices Second Edition; Tentative Guideline (1992).** Offers guidelines for designing an experiment to evaluate the precision performance of clinical chemistry devices; recommendations on comparing the resulting precision estimates with manufacturer's precision performance claims and determining when such comparisons are valid; and manufacturer's guidelines for establishing claims.

EP9-A **Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline (1995).** This document addresses procedures for determining the bias between two clinical methods or devices and design of a method comparison experiment using split patient samples and data analysis.

H3-A4 **Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard Fourth Edition(1998).** This document provides provedures for the collection of blood specimens by venipuncture, including line draws, blood culture collection, and venipuncture in children. Includes recommendations on order of draw.

H5-A3 **Procedures for the Handling and Transport of Diagnostic Specimens and Etiologic Agents Third Edition; Approved Standard (1994).** American National Standard. Gives proper packaging, handling, and transport requirements for medical specimens and governing regulations.

M29-A **Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Approved Guideline (1997).** Provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in the laboratory setting; specific precautions for preventing the laboratory transmission of bloodborne infection from laboratory instruments and materials; and recommendations for the management of bloodborne exposure.

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

NCCLS ▼ 940 West Valley Road ▼ Suite 1400 ▼ Wayne, PA 19087 ▼ USA ▼ PHONE 610.688.0100
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