



## Laboratory Procedure Manual

*Analyte:* **Iron and TIBC**

*Matrix:* **Serum**

*Method:* **Modification of the automated AAll-25  
colorimetric method**

*Method No.:*

*Revised:*

*as performed by:* *Inorganic Toxicology and Nutrition Branch  
Division of Laboratory Sciences  
National Center for Environmental Health, CDC*

*Contact:* *Dr. Eric J. Sampson, Director  
Division of Laboratory Sciences*

### **Important Information for Users**

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

## Public Release Data Set Information

This document details the Lab Protocol for NHANES 1999–2000 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
lab06	LBXIRN	Iron ( $\mu\text{g/dL}$ )
	LBDIRNSI	Iron ( $\mu\text{mol/L}$ )
	LBXTIB	Total iron binding capacity (TIBC) ( $\mu\text{g/dL}$ )
	LBDTIBSI	Total iron binding capacity (TIBC) ( $\mu\text{mol/L}$ )

## 1. Summary of Test Principle and Clinical Relevance

Serum iron and total iron-binding capacity (TIBC) are measured by a modification of the automated AAII-25 colorimetric method, which is based on the procedures of Giovaniello et al. (1) and of Ramsey (2). The method has been modified further to be performed on an Alpkem Flow Solutions IV (rapid-flow analysis) system. Iron is quantitated by measuring the intensity of the violet complex formed in the reaction between ferrozine and  $\text{Fe}^{++}$  in acetate buffer at 562 nm. Thiourea is added to complex  $\text{Cu}^{++}$ , which can also bind with ferrozine and yield falsely elevated iron values. In TIBC tests, serum is mixed with 400  $\mu\text{g}/\text{dL}$  iron solution to saturate the iron-binding sites of the serum transferrin molecules. Magnesium carbonate is used to remove excess iron. Centrifugation is used to precipitate the magnesium carbonate, and the supernatant is measured for iron content.

Serum iron and TIBC assays can be used together with ferritin assays to aid in the diagnosis of iron deficiency or overload. In cases of iron deficiency, decreased serum iron levels and increased TIBC may be observed. Conversely, in cases of iron overload (which can be genetic as idopathic hereditary hemochromatosis, or can be caused by hemolytic anemia, liver damage, excessive absorption of iron, and iron therapy) increased serum iron levels and decreased TIBC may be observed. In cases of infection, inflammation, and malignancy, both serum iron levels and TIBC may be decreased (3).

## 2. Safety Precautions

Treat all serum specimens as potentially positive for HIV, and hepatitis B and C. Therefore, observe Universal Precautions. Wear gloves, lab coat, and safety glasses at all times during the analysis. We recommend the hepatitis B vaccination series for all analysts working with whole blood or serum samples. When the analysis is completed, dispose of all specimens and all plastic and glassware materials coming in contact with specimens by autoclaving. Several of the reagents for this method are corrosive or caustic and should be handled appropriately. Observe proper laboratory safety guidelines with respect to pipetting, reagent preparation, etc.

Reagents and solvents used in this study include those listed in Section 6. Material safety data sheets (MSDS) for these chemicals are readily accessible as hard copies in the lab. If needed, MSDS for other chemicals can be viewed at <http://www.ilpi.com/msds/index.html> or at <http://intranet.cdc.gov/ohs>.

## 3. Computerization; Data System Management

- A. Calculation and statistical evaluation of a given run is accomplished with the Microsoft Excel software installed on the PC. After a run is complete and any additional corrections by the analyst are made, the result file (containing the patient data as well as the QC data) is electronically transferred to the appropriate analyte-specific subfolder in Q:/ITN/Nutrition Lab/Import into Access on the NCEH/DLS Local Area Network (LAN). The analyst also gives a hardcopy of the result file to the reviewing supervisor. After the reviewing supervisor approves the final values for release by checking off the bench and blind QC values and signing the hardcopy, he/she sends an email to the computer support staff that the data has been released to be imported into the NHANES 1999+ database that is located in Microsoft Access; the computer support staff imports the data into the NHANES 1999+ database by using a macro. Data entry is verified by the computer support staff and the supervisor. Data is transmitted electronically several times weekly to Westat's ISIS computer system, and transferred from there to NCHS. Abnormal values are confirmed, and codes for missing data are entered by the analyst and are transmitted as part of the data file to the Westat ISIS computer, and are eventually forwarded to NCHS. Westat also prepares the abnormal report notifications for the NCHS Survey Physician.
- B. Files stored on the network or CDC mainframe are automatically backed up nightly by DLS LAN support staff and CDC Data Center staff, respectively. Backup of the daily data containing all raw data files and result files for each run are the responsibility of the analyst. Typically these files are backed up once a week onto a floppy disk or a CD-ROM using a CD writer.

- C. Documentation for data system maintenance is contained in printed copies of data records, as well as in "system log" files on the local hard drives used for the archival of data.

4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

- A. For best results, a fasting sample should be obtained.
- B. Specimens for iron / TIBC analysis may be fresh or frozen serum, harvested from blood collected in a red-top (no anticoagulant) evacuated blood collection tube, by standard venipuncture procedures.
- C. A 1-mL sample of serum is preferable; a minimum sample volume of 500  $\mu$ L is required for both iron and TIBC analyses.
- D. The appropriate amount of serum is dispensed into a 2.0-Nalge cryovial or other plastic screw-capped vial labeled with the participant's ID.
- E. Specimens collected in the field are frozen and then shipped on dry ice by overnight mail. Serum iron and TIBC are very stable and specimens may be stored at  $-20$  to  $-70^{\circ}\text{C}$  for years. Serum iron levels are not affected by freeze-thaw cycles; after two cycles, TIBC values may tend to be reduced.
- F. Specimens generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site of collection.
- G. Because a certain amount of diurnal variation is associated with the levels of metals in the body, the time of collection should be noted for the NHANES data analysis. Iron background contamination levels are lower in the red-top collection tubes, whereas the royal blue-top "trace metals-free" tubes are more appropriate for zinc and copper specimen collection. Hemolyzed specimens should not be used because of the contribution of iron from hemoglobin.
- H. Specimen handling conditions are outlined in the Policies and Procedures Manual of DLS (copies are available in the Nutritional Laboratory and the electronic copy of this file is located at Q:/ITN/Nutrition Laboratory/CLIA). The protocol discusses collection and transport of specimens and the special equipment required. In general, plasma should be transported and stored at no more than  $-20^{\circ}\text{C}$ . Samples thawed and refrozen less than five times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood or plasma should be transferred into a sterile Nalge cryovial labeled with the participant's ID.

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable for this procedure

6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

A. Reagent Preparation

- (1) 0.2 mol/L hydrochloric acid with 3 g/dL sodium chloride  
To 250 mL of deionized water in a 2-L flask, add 34 mL concentrated HCl and 60 g NaCl. Mix well and dilute to volume with deionized water. (Prepare as needed to be used as part of the working solution; this solution is stable at  $20$ – $25^{\circ}\text{C}$ .)
- (2) Working HCl/NaCl/Brij solution  
For each 150 samples to be analyzed, add 20 drops Brij to 200 mL of 0.2 mol/L HCl with 3 g/dL NaCl. Mix well. (Prepare daily.)
- (3) Working HCl/NaCl/ascorbic acid solution  
For each 150 samples to be analyzed, add 1 g of L-ascorbic acid and 10 drops Brij to 100 mL of 0.2 mol/L HCl with 3 g/dL NaCl. Mix well. (Prepare daily.)

- (4) 0.75 mol/L acetate buffer  
Add 408.4 g sodium acetate to 2 L deionized water in a 4-L flask. Stir well and dilute to volume (Prepare weekly; the solution is stable at 20–25°C.)
  - (5) 0.07 g/dL ferrozine with 1% (w/v) thiourea  
Add 0.7 g Ferrozine and 10 g thiourea to 1 L of acetate buffer-thiourea solution and mix well. Filter through a 0.45- $\mu$ m Millipore filter to remove any undissolved particulate matter. (Prepare weekly; the solution is stable at 20–25°C.)
  - (6) 0.5 ml/L Brij-35 wash solution (v/v)  
Add 1.0 mL Brij-35, 30% solution, to 2 L deionized water and mix well. (Prepare weekly.)
  - (7) 0.1 mol/L hydrochloric acid (for standard preparation)  
Add 8.3 mL concentrated HCl to 500 mL deionized water in a 1-L volumetric flask. Mix well and dilute to volume with additional water. Do not add Brij-35. (Approximately 5 L of this solution is required to prepare intermediate and working standards.)
  - (8) 400  $\mu$ L/dL iron saturating solution for TIBC  
Dilute 2.0 mL of the 1.0 g/L stock iron standard to volume in a 500-mL flask with deionized water. (Transfer to plastic storage bottle and allow to stabilize for one week before use. The solution is stable at 20–25°C.)
- B. Standards Preparation (allow standards to equilibrate for 24–72 hours)
- (1) g/L stock iron standard solution  
Place 1.000 g iron wire in a 1-L volumetric flask. Add 12 mL concentrated HCl and dissolve the wire with slight warming. After the wire is completely dissolved, cool the flask to room temperature and dilute contents to volume with deionized water. (The solution is stable indefinitely; store in a polypropylene container at 20–25°C.)
  - (2) 50.0 mg/L iron intermediate stock solution  
Dilute 25 mL of the 1.0 g/L stock iron solution to 500 mL with 0.1 mol/L HCl. (Prepare each time new working standards are required.)
  - (3) Working iron standards  
In a series of 500-mL volumetric flasks, prepare dilutions from the intermediate standard as shown below. Dilute to 500 mL with 0.1 mol/L HCl and mix well. (Prepare as needed; the solution is stable at 20–25°C.)
  - (4) NIST iron standard (SRM 937)  
This standard reference material has a concentration of 1.0001 mg/mL and may be diluted at concentrations from 1 to 1000  $\mu$ L/dL as a verification of the accuracy of the working standard dilutions.
- C. Preparation of Quality Control Materials
- High-quality serum from fasting human subjects, confirmed to be negative for antibodies to HIV, hepatitis B, and hepatitis C, is used for the preparation of quality control pools. A base normal pool is prepared, and the levels of iron and TIBC are measured for reference. A portion of the base pool is diluted 25–30% with sterile physiological saline to reduce levels to low-normal. Another aliquot is subjected to filtration on a hollow-fiber column, which removes about 30% of the water content of the serum and concentrates the metals as well as the serum proteins. Care is taken to ensure that total serum proteins do not exceed 8 g/dL. This technique has been used effectively to provide simultaneously high levels of iron and high TIBC. All three pools are filter-sterilized (through 0.22- $\mu$ m filters), and then dispensed into glass Wheaton vials, which are capped and stored at –70°C for maximum stability.

See Section 10, Quality Control Procedures, for target values of quality control pools.

Two levels (low-normal and high-normal ferritin concentrations) of blind QC pools may be prepared from pooled, filter-sterilized human serum obtained from fasting donors with elevated or decreased ferritin levels. Pool serum in acid-cleaned 20-L glass carboys. Mix well on a magnetic stirrer. Clean-filter the serum through in a sequential manner using filters of the following pore sizes, each preceded by a pre-filter: 3.00  $\mu\text{m}$ , 1.20  $\mu\text{m}$ , 0.80  $\mu\text{m}$ , 0.65  $\mu\text{m}$ , 0.45  $\mu\text{m}$ , 0.30  $\mu\text{m}$ , and 0.22  $\mu\text{m}$ .

Through the use of sterile technique under a laminar-flow hood, dispense the serum in 1-mL aliquots with a Micromedic Digiflex dispenser into 2.0 mL Nalge cryovials. Cap and label the vials with NHANES bar-coded labels that have been specially prepared for the QC pools. Store the pools at  $-70^{\circ}\text{C}$  at the CDC CASPIR Specimen Repository in Lawrenceville where they will be inserted randomly into the NHANES runs. Select 20 vials of each level.

#### D. Other Materials

- (1) 0.25-mL disposable conical bottom polystyrene sample cups (Baxter Scientific Products., McGaw Park, IL).
- (2) Disposable filtering columns (Whale Scientific Co., Denver, CO).
- (3) "Ferrozine" iron reagent [3-(2 pyridyl)-5,6 bis (4-phenylsulfonic acid)-1, 2, 4, triazine, monosodium, monohydrate], 95% purity (Hach Chemical Co., Ames, IA).
- (4) L-ascorbic acid, 99.9% purity (J.T. Baker Chemical Co., Phillipsburg, NJ).
- (5) Magnesium carbonate (basic), "Fisher Certified" (Fisher Chemical Co.).
- (6) Thiourea, "Baker Analyzed" (J.T. Baker).
- (7) Sodium acetate, trihydrate, "Baker Analyzed" (J.T. Baker).
- (8) Hydrochloric acid (HCl), concentrated, reagent grade (J.T. Baker).
- (9) Brij-35, 30% solution (Pierce Chemical Co, Rockford, IL).
- (10) 12- x 75-mm disposable glass culture tubes (Corning Glass Works, Corning, NY) lot-tested for iron contamination.
- (11) Iron wire, 99.9% purity (Mallinckrodt Chemical Works, St. Louis, MO).
- (12) Deionized water, 1.0 M $\Omega$  cm at 25 $^{\circ}\text{C}$  (Continental Water Co., Atlanta, GA).
- (13) Sodium chloride (NaCl), ACS certified (Fisher Scientific Co.).
- (14) Kemwash detergent solution (Alpkem, Inc.).
- (15) 0.45  $\mu\text{m}$  filters (Millipore, Bedford, MA)

#### E. Instrumentation

- (1) Alpkem Flow Solutions (III/IV) 3000 Analyzer system (Alpkem, Inc., College Station, TX).
  - (a) Model RA sampler.
  - (b) Model 509 power module, model 502 mini-pump and dialyzer with type "H" membrane and flow-rated tubing.
  - (c) Model ER detector, with 15-mm flowcell and 562-nm filters
  - (d) Data system, with WinFlow V3 software, Pentium computer, and printer.
- (2) Micromedic Digiflex automatic dispenser equipped with 2-mL sampling and dispensing syringes (Titertek, Inc. Huntsville, AL).
- (3) Vortex mixer (Fisher Scientific Co., Fairlawn NJ).
- (4) IEC Centra GP-8 centrifuge (International Equipment Co., Needham Heights, MA).

## 7. Calibration and Calibration Verification Procedures

The accuracy and precision of iron measurements was verified with NIST SRM 909, multi-element serum reference material, 2.34  $\mu\text{L}/\text{mL}$  or 234  $\mu\text{L}/\text{dL}$ . NIST stocks of SRM 909 have been exhausted; its replacement material is SRM 909b. Accuracy and precision were also verified with NIST iron standard SRM 937, iron metal in HCl, 99.9% purity. The latter standard reference material has a concentration of 1.0001 mg/mL and may be diluted at concentrations such as 1, 5, 10, 50, 100, 500, and 1000  $\mu\text{L}/\text{dL}$  in order to verify the accuracy of the working standard dilutions. Linearity of the method may also be confirmed with the same dilutions of SRM 937. NIST SRM 2124-3, iron standard solution, 10 mg/mL, may also be diluted and used for these purposes. In each case, at least three replicates of each dilution are run as unknowns against working standards, with a linear regression generated from working standards as X and NIST dilutions as Y. The correlation coefficient should be 0.98 or higher, the slope should be  $1.00 \pm 0.05$ , and the y-intercept should be  $0.0 \pm 1.0$ . This procedure should be run twice a year on each operating system used for this method, with common dilutions run on each system.

Additionally, each time new batches of working standards are prepared from stock, "new" dilutions are analyzed against "old" dilutions for accuracy and linearity, with the same criteria of acceptability required before the new batches are implemented in the assay. New standard values should be within 2% of old standard values.

The Alpkem systems are maintained under a service contract with O-I Analytical (Alpkem's parent company); this contract calls for twice-yearly preventive maintenance to test calibration parameters against manufacturing specifications.

## 8. Procedure Operating Instructions; Calculations; Interpretation of Results

### A. Procedure

#### (1) Preparation of samples for serum iron assay:

Mix serum specimens well. Filter about 0.3 mL of serum into a 0.5-mL sample cup, using a Whale Scientific disposable plastic filtration column to remove fibrin and any other cellular debris.

#### (2) Preparation of samples for TIBC assay:

Using the Micromedic automatic pipette, dilute 0.2 mL well-mixed serum with 0.4 mL 400  $\mu\text{L}/\text{dL}$  iron saturating solution in 12- × 75-mm tubes. Mix well by vortexing and allow the tubes to stand for at least 30 min. (We have found that results are most precise when the samples are tightly capped and kept at 4–8°C overnight. They may also be stored at –20°C for up to 3 weeks.) Add 0.1 g basic magnesium carbonate directly to each tube of diluted serum. Mix the contents of the tubes; allow the tubes to stand for 45 min, mixing at 15-min intervals. Centrifuge the tubes at 2500 × g for 10 min to pack the magnesium carbonate. Filter the supernatant into 0.25-mL sample cups and proceed as with the iron analysis.

### B. Quality Control Materials

Assay frozen serum quality control materials in the same manner as serum specimens.

NOTE: Past publications have stated that TIBC cannot be accurately determined on lyophilized, commercial, quality control serum products or materials such as the College of American Pathologists (CAP) materials. However, in 1998, CAP agreed to a test evaluation of TIBC levels in their lyophilized materials (for native TIBC levels of their base pools) in order to develop a tentative proficiency testing program for this analyte.

### C. Operation

Follow standard operating protocol as outlined in the Operations Guide for the Alpkem Flow Solutions 3000 (4). Approximately 100  $\mu\text{L}$  of serum is required for each iron analysis, and 200  $\mu\text{L}$  serum is used for the TIBC dilution. The analysis rate is 72 samples per hour. Calibration is accomplished with

ascending- and descending-order standard curves analyzed with every 60–80 specimens. At maximal capacity, an ascending-order standard curve, 4 racks of 20 specimens each, and a descending-order standard curve can be run in one session. Two sessions can be run in a single day, one in the morning and a second in the afternoon. Quality control materials are included with every curve and every rack of 20 specimens analyzed. With full daily use of the system, pump-tubing and type "H" membranes should be changed every 5 days. Using Windows-based software helps analysts to monitor any unusual changes in peak size or shape, which may indicate, baseline shifts, or dialysis or line clogging problems. Full scale on the monitor is 0.1A. For maximum sensitivity, the system is peaked with each session with the 300 µL/dL standard.

#### D. CDC Modifications

CDC has modified the Technicon AAII-25 method in the following ways (5):

- (1) The reagent concentrations used and their ratios are based on procedures developed at CDC.
- (2) The use of type "H" rather than type "C" membranes was developed in conjunction with Alpkem in order to provide maximum efficiency of dialysis.
- (3) Ferrozine as well as thiourea is incorporated into the acetate buffer.
- (4) A 15- rather than a 10-mm stainless steel end-capped flowcell is used in the photometer to enhance sensitivity.

#### E. Special Method Notes

Because of the highly enhanced sensitivity of this method, developed to minimize the amount of sample required so that pediatric specimens could be used, it is very important to eliminate all sources of particulate matter or specimen fibrin that could clog the micro-sized pump tubing. We therefore recommend that the chart recorder be used at all times, if possible, as a visual aid to dialysis performance. The system should be washed weekly by pumping HCL for 10 minutes followed by water for 10 minutes, followed by NaOH for 10 minutes through all tubes to prevent protein buildup. An additional washing using water for 30 min can be done to ensure that all chemicals and protein have been flushed from the system. The flowcell should be removed from the sample stream at this time to prevent etching of its glass interior.

### 9. Reportable Range of Results

The serum iron concentrations of specimens and the TIBC of diluted samples are calculated from the slope and y intercept of the 8-point regression line of the expected concentrations of the standards versus their millivolt recorder values (or absorbance values).  $R^2$  for the regression line should be  $>0.9990$ . The WinFlow data system will generate calculations for the run. TIBC concentrations are multiplied by the dilution factor of 3. Percent saturation, which is the estimate of iron-filled available binding sites on the transferrin molecule, is expressed as iron/TIBC.

The method is linear from 0 to 1000 µL/dL, as verified by successive dilutions of NIST SRMs. Specimens with iron values  $<30$  µL/dL or  $>200$  µL/dL, and TIBC  $<250$  µL/dL or  $>500$  µL/dL are reanalyzed for confirmation. Percent recovery was established as 99.3% over the linear range by using the method of standard additions. The limit of detection is approximately 2.0 µL/dL. The average total CV over the reporting range, as demonstrated by QC pools, is approximately 3%.

### 10. Quality Control (QC) Procedures

#### A. Blind Quality Controls

Blind QC specimens are inserted prior to the arrival of the samples in the Inorganic Toxicology and Nutrition Branch. These specimens are prepared at two levels so as to emulate the patient samples;

the labels used are identical to those used for patient samples. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 specimens analyzed.

#### B. Bench Quality Controls

Bench QC specimens are prepared from three plasma pools, which represent low, intermediate, and high levels of MMA in plasma. These pools are prepared in the same manner as patient samples and analyzed in duplicate as part of each run.

The results from the pools are checked after each run. The system is declared "in control" if all three QC results are within 2s limits and the run is accepted. If one of the three QC results is outside the 2s limits then apply rules below and reject if any condition is met - the run is then declared "out of control":

1<sub>3s</sub> Any of the three QC results are outside the 3s limit

2<sub>2s</sub> Two of the three QC results in the run are outside the 2s limit (same side of mean)

R<sub>4s</sub> Sequential QC results (either within the run or across runs) are outside the 2s limit on the opposite sides of the mean

10<sub>x</sub> Ten sequential QC results (across pools and across runs) are on the same side of the mean

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared "out of control" for that analyte as assessed by internal (bench) QC.

The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated quarterly. When necessary, limits are updated to include more runs.

While a study is in progress, electronic copies of the QC results from each run are stored in the analyte-specific folder on Q:/ITN/Nutrition Lab/Data handling/Import into Access. Electronic copies of the tracking of the QC results over time are stored in the analyte-specific folder on Q:/ITN/Nutrition Lab/Data handling/QC Results in Excel. A hardcopy of the QC results from each run is also kept by the analyst.

#### 11. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

If the system should be declared "out of control," take the following remedial action(s):

- (1) Check the system for fibrin clots in sample or metal connectors.
- (2) Replace the type "H" membrane.
- (3) Replace the pump tubing.
- (4) Check timing and bubble gating.

After troubleshooting procedures have been completed and the system has been verified to be "in control," re-analyze all specimens for that analytical run and report the values from the re-analysis rather than the original values.

12. Limitations of Method; Interfering Substances and Conditions

TIBC levels may tend to be reduced in samples that have undergone more than two freeze-thaw cycles. Background contamination levels of iron are lower in red-top Vacutainers than in blue-top Vacutainers. Hemolyzed specimens should not be used because of the contribution of iron from hemoglobin.

13. Reference Ranges (Normal Values)

Values for adult males (>20 years old) are generally higher than those for adult females, and children have lower values than adults. Low iron values in conjunction with elevated TIBC values, yielding <15% saturation, are generally indicative of iron deficiency anemia. Elevated iron values, in conjunction with >60% saturation and an elevated ferritin level, may be indicative of hemochromatosis, or iron overload. These are the normal ranges for the U.S. population based on the NHANES II data (6):

- Males >18 years old: 60-190  $\mu\text{L/dL}$  iron, 300-455  $\mu\text{L/dL}$  TIBC
- Females >18 years old: 40-175  $\mu\text{L/dL}$  iron, 285-510  $\mu\text{L/dL}$  TIBC
- Children 3-17 years old: 32-175  $\mu\text{L/dL}$  iron, 305-490  $\mu\text{L/dL}$  TIBC

14. Critical Call Results (Panic Values)

Iron values <30  $\mu\text{L/dL}$  or <15% saturation are considered indicative of possible iron deficiency. Transferrin saturation >55% in combination with a ferritin value >300 ng/mL may be indicative of possible iron overload, or hemochromatosis. No critical call results are defined for this method because of the epidemiological nature of the survey.

15. Specimen Storage and Handling During Testing

Allow specimens to gradually reach room temperature before sample preparation and during testing.

16. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

Since the analysis of serum for iron and TIBC is inherently complex and challenging, there are no acceptable alternative methods of analysis in the NHANES laboratory. If the analytical system fails, we recommend storing the specimens at  $-20^{\circ}\text{C}$  until the analytical system is restored to functionality.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)

The collaborating agency with access to patient identifiers or the responsible medical officer is notified by FAX by the supervisor of any iron result that is <30  $\mu\text{L/dL}$  or transferrin saturation <15%, which are considered indicative of possible iron deficiency. They are also notified by FAX of transferrin saturation results >55% in combination with a ferritin value >300 ng/mL, which may be indicative of possible iron overload or hemochromatosis. Copies of Faxes sent concerning abnormal results are kept in a notebook by the supervisor for the duration of the study.

Test results that are not abnormal are reported to the collaborating agency at a frequency and by a method determined by the study coordinator. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as an ASCII text file or Excel file, either through electronic mail or on a diskette.

For NHANES 1999+, all data are reported electronically several times weekly to the Westat ISIS computer and then are transferred to NCHS. For some smaller studies, hard copies of a data report are sent, as well as the results in electronic format.

#### 18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

The Microsoft Access database is used to keep records and track specimens for NHANES 1999+. If plasma or serum methylmalonic acid analyses are used for smaller, non-NHANES studies, records are kept on files in Q:\ITN\Nutrition Lab on the DLS LAN.

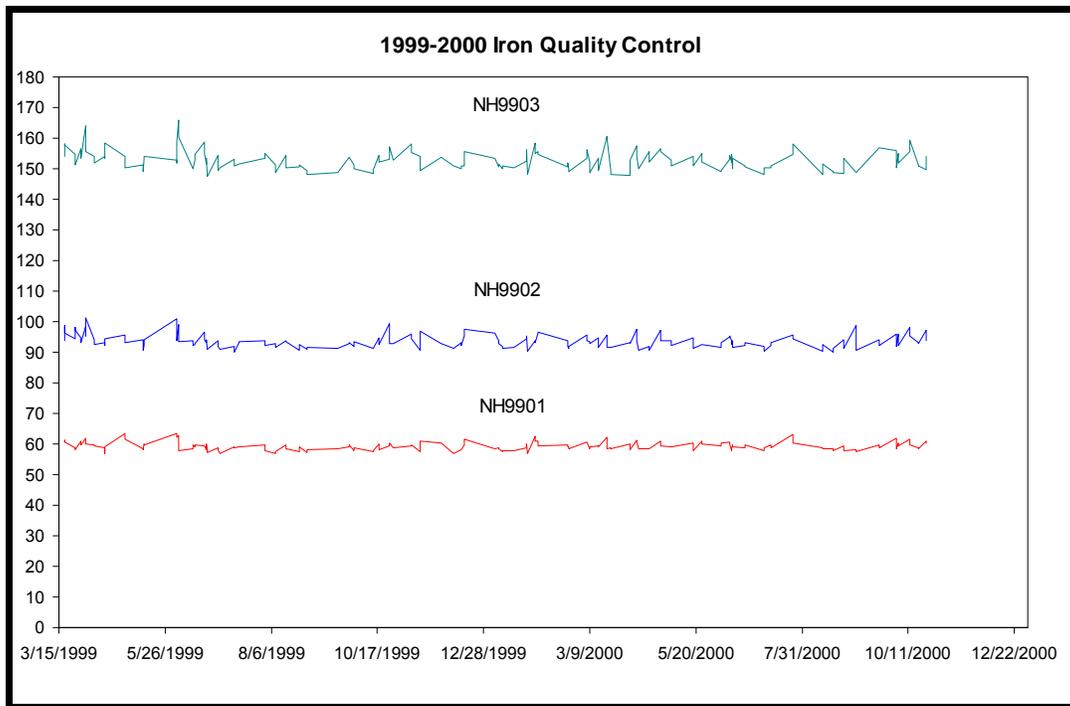
We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the NHANES study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual serum from these analyses for non-NHANES studies may be discarded at the request of the principal investigator, or may be transferred to the CDC CASPIR facility for use by other investigators. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens stored in a freezer at  $-70^{\circ}\text{C}$ . The specimen ID is read off of the vial by a barcode reader attached to the computer used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the DIF file containing the electronic copy of the results is loaded into the database, and the analytical results are linked to the database by ID number. The analyst is responsible for keeping a notebook containing the ID numbers of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies.

19. Summary Statistics and QC Graphs

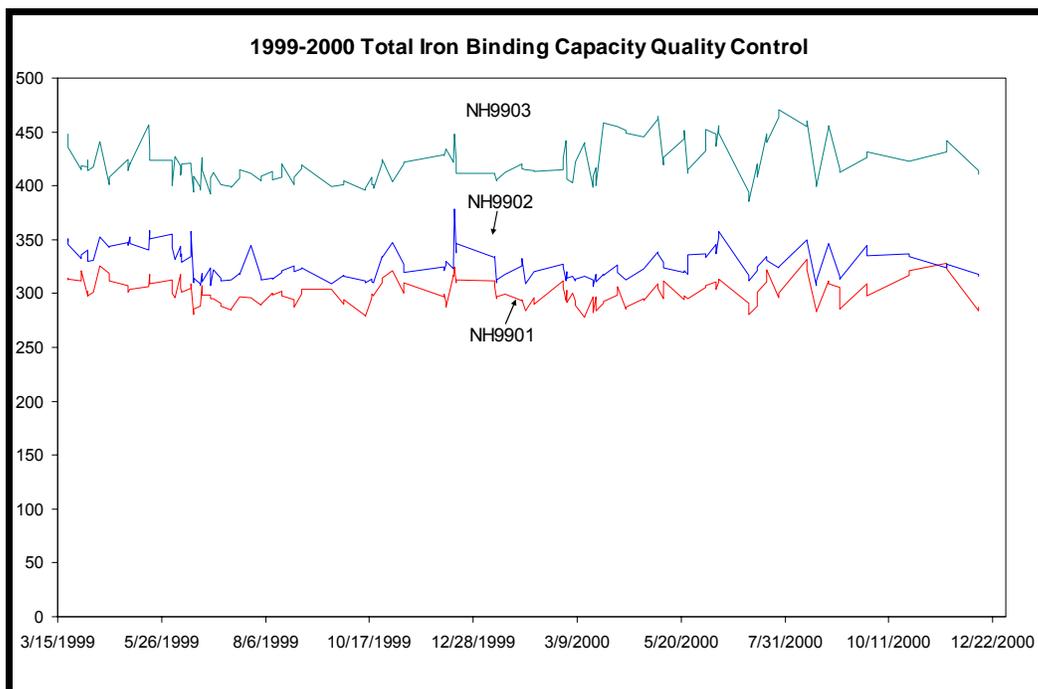
A. Iron

Summary Statistics for Iron by Lot						
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
NH9901	153	3/19/1999	10/23/2000	59.3	1.4	2.4
NH9902	154	3/19/1999	10/23/2000	93.6	2.2	2.4
NH9903	153	3/19/1999	10/23/2000	152.9	3.4	2.2



B. TIBC

Summary Statistics for Total Iron Binding Capacity by Lot						
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
NH9901	147	3/22/1999	12/12/2000	300.56	11.53	3.84
NH9902	147	3/22/1999	12/12/2000	326.88	14.18	4.34
NH9903	146	3/22/1999	12/12/2000	421.68	18.90	4.48



## References

1. 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.
2. Ramsey WNM. The determination of the total iron-binding capacity of serum. *Clin Chem Acta* 1957; 2:221.
3. Gibson RS. Principles of nutritional assessment. New York: Oxford University Press, 1990
4. FLOW SOLUTIONS 3000 system operations manual. Wilsonville, OR: Alpkem, Inc., 1997.
5. 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.
6. Fulwood R, Johnson CL, Bryner JD, et al. Hematological and nutritional reference data for persons 6 months - 74 years of age; United States, 1976-80. Vital and Health Statistics. Series 11 - No. 232. DHHS Pub. No. (PHS) 83-1682. Washington, D.C.: Public Health Service, 1982.
7. Gunter EW, Lewis BL, Koncikowski SM. Laboratory methods used for the Third National Health and Nutrition Examination Survey (NHANES III), 1988-1994. Centers for Disease Control and Prevention, Hyattsville, MD, 1996.

## Additional Sources

1. Van Assendelft OW, Fairbanks VF, Gunter EW, Turner WE. Proposed standard for the determination of serum iron and total iron-binding capacity. Provisional Standard, National Committee for Clinical Laboratory Standards, 1995.
2. Looker AC, Gunter EW, Johnson CL. Methods to assess iron status in various NHANES Surveys. *Nutrition Reviews*, 53:9, 246-54, 1995.
3. Looker AC, Dallman PR, Carroll MD, Gunter EW, Johnson CL. Prevalence of iron deficiency in the U.S. *Journal of the American Medical Association*, 277:973-976; 1997.
4. Gambino R, Desvarieux E, Ort M, Matan H, Ackattaathil, Lijoi E, Wimmer C, Bowers J, Gunter E. The relation between chemically measured total iron-binding capacity levels and immunologically measured transferrin levels in human serum. *Clinical Chemistry*, 43(12);2808-12, 1997.
5. Gunter EW, McDonnell SM. Reply to Letter to the Editor, "Transferrin saturation and screening of genetic hemochromatosis." *Clinical Chemistry*, 44/2:361-2, 1998.
6. Gunter EW, van Assendelft OW, Meyers GL, Caudill SP. A pilot interlaboratory comparison of methods for measurement of serum iron, total iron-binding capacity, and ferritin: Is there a need for standardization? *Mayo Clinic Proceedings*, submitted, December 1998.

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**Appendix (Tables 1-3)**

**Table 1. Working Iron Standards**  
(Dilute to 500 mL with 0.1 mol/L HCl)

50 mg/L Intermediate Standard, mL	Final Concentration, µL/dL Iron
3	30
5	50
8	80
10	100
15	150
20	200
25	250
30	300

**Table 2. Typical Serum Iron Quality Control Pool Results (From NHANES III)**

Pool	Mean	95% limits	99% limits	N	Total SD	Total CV
0888	69.83	65.93-73.73	64.70-74.96	102	2.84	2.84
0988	88.12	84.11-92.14	82.84-93.41	102	3.33	2.93
1088	138.30	131.76-145.85	129.53-148.08	102	3.23	4.63
9114	73.80	70.75-76.81	67.79-77.77	33	2.11	2.86
9115	98.19	94.55-101.84	93.40-102.99	33	2.62	2.57
9116	143.31	139.23-147.40	137.94-148.69	33	2.80	1.95

**Table 3. Typical Serum Total Iron-binding Capacity Quality Control Pool Results (From NHANES III)**

Pool	Mean	95% limits	99%limits	N	Total SD	Total CV
0888	285.6	266.02-305.09	259.84-311.27	92	11.97	4.19
0988	370.7	338.92-401.53	330.18-411.27	92	17.42	4.70
1088	496.6	465.87-527.90	456.06-537.71	92	18.51	3.73
9114	260.5	243.80-277.23	238.51-282.52	33	9.13	3.50
9115	369.2	346.55-391.85	339.39-399.01	33	12.87	3.49
9116	502.11	474.26-529.96	464.45-538.77	33	16.04	3.20