Laboratory Procedure Manual

Analyte: **Total Cholesterol, HDL-Cholesterol, Triglycerides, and LDL-Cholesterol**

Matrix: **Serum**

Method: **Hitachi 704 Analyzer**

Method No.:

Revised:

as performed by: *Lipoprotein Analytical Laboratory*
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Public Release Data Set Information

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

The data for these methods can be found in two separate files. Lab13 contains the data for total cholesterol and HDL. Lab13am contains the data for triglycerides and LDL.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
lab13	LBXTC	Total cholesterol (mg/dL)
	LBDTCSI	Total cholesterol (mmol/L)
	LBXHDL	Precipitated HDL-cholesterol (mg/dL)
	LBDHDLSI	Precipitated HDL-cholesterol (mmol/L)
	LBXHDD	Direct HDL-cholesterol (mg/dL)
	LBDHDDSI	Direct HDL-cholesterol (mmol/L)
lab13am	LBXTR	Triglycerides (mg/dL)
	LBDTRSI	Triglycerides (mmol/L)

1. SUMMARY OF TEST PRINCIPLES AND CLINICAL RELEVANCE

A. TOTAL CHOLESTEROL

Cholesterol is measured enzymatically in serum or plasma in a series of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3-OH group of cholesterol. One of the reaction byproducts, H_2O_2, is measured quantitatively in a peroxidase catalyzed reaction that produces a color. Absorbance is measured at 500 nm. The color intensity is proportional to cholesterol concentration. The reaction sequence is as follows:

$$\text{cholesteryl ester} + H_2O \xrightarrow{\text{cholesteryl ester hydrolase}} \text{cholesterol} + \text{fatty acid}$$

$$\text{cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{4-(}p\text{-benzoquinone-monoimino)-phenazone} + 4\ H_2O$$

Elevated levels of cholesterol increase the risk for coronary heart disease (CHD). Cholesterol is measured to help assess the patient's risk status and to follow the progress of patient's treatment to lower serum cholesterol concentrations. Desirable cholesterol levels are considered to be those below 200 mg/dL in adults and below 170 mg/dL in children.

B. TRIGLYCERIDES

Triglycerides are measured enzymatically in serum or plasma using a series of coupled reactions in which triglycerides are hydrolyzed to produce glycerol. Glycerol is then oxidized using glycerol oxidase, and H_2O_2, one of the reaction products, is measured as described above for cholesterol. Absorbance is measured at 500 nm. The reaction sequence is as follows:

$$\text{Triglycerides} + 3H_2O \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerokinase}} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{glycerophosphate oxidase}} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{peroxidase}} \text{4-(}p\text{-benzoquinone-monoimino)-phenazone} + 2H_2O + HCl.$$

High levels of serum triglycerides help mark conditions that are associated with increased risk for CHD and peripheral atherosclerosis. High triglycerides are associated with increased risk for CAD in patients with other risk factors, such as low HDL-cholesterol, some patient groups with elevated apolipoprotein B concentrations, and patients with forms of LDL that may be particularly atherogenic. Desirable fasting triglyceride levels are considered to be those below 200 mg/dL, and are further categorized as Borderline, 200–400 mg/dL; High, 400–1,000 mg/dL; and Very High (> 1000 mg/dL).

Very high triglycerides can result in pancreatitis and should be promptly evaluated and treated. Triglycerides are also measured because the value is used to calculate low density lipoprotein (LDL)-cholesterol concentrations (see below). In NHANES 1999–2000, triglyceride is only measured in specimens from fasting participants, i.e., those sampled in Session 1.

C. HIGH DENSITY LIPOPROTEIN (HDL) CHOLESTEROL

In NHANES 1999–2000, HDL-cholesterol is measured in two ways: following the precipitation of the other lipoproteins with a polyanion-divalent cation mixture; and directly in serum without the need to remove the apoB-containing lipoproteins (see below). The heparin-Mn^{+2} method is used for all specimens except those from 3–5 year old participants. The direct method is used for that group because of the limited sample volumes available from young children.

Low serum concentrations of HDL-cholesterol are associated with increased risk for CHD. Coronary risk increases markedly as the HDL concentration decreases from 40 to 30 mg/dL. A low HDL-cholesterol concentration is considered to be a value below 35 mg/dL, and high HDL, ≥ 60 mg/dL. HDL-cholesterol values are also used in the calculation of LDL-cholesterol (see LDL section below).

(1) Heparin-Mn^{+2} method. ApoB containing lipoproteins are removed by precipitation with heparin sulfate and MnCl2 and cholesterol is measured in the HDL-containing supernatant The precipitation procedure for samples and controls is detailed in Section 10.f. HDL-cholesterol is measured in the clear supernatant. Cholesterol in the HDL-containing fraction is measured as described above for total cholesterol.

(2) Direct HDL method. HDL is measured directly in serum. The basic principle of the method is as follows. The apoB containing lipoproteins in the specimen are reacted with a blocking reagent that renders them non-reactive with the enzymatic cholesterol reagent under conditions of the assay. The apoB containing lipoproteins are thus effectively excluded from the assay and only HDL-chol is detected under the assay conditions.

The reagents are purchased from Roche/Boehringer-Mannheim Diagnostics. The method uses sulfated alpha-cyclodextrin in the presence of Mg^{+2}, which forms complexes with apoB containing lipoproteins, and polyethylene glycol-coupled cholesteryl esterase and cholesterol oxidase for the HDL-cholesterol measurement. The reactions are as follows:

ApoB containing lipoproteins + α-cyclodextrin + Mg^{+2} + dextran SO_4 $\longrightarrow$ soluble non-reactive complexes with apoB-containing lipoproteins

HDL-cholesteryl esters $\xrightarrow{\text{PEG-cholesteryl esterase}}$ HDL-unesterified cholesterol + fatty acid

unesterified chol + O_2 $\xrightarrow{\text{PEG-cholesterol oxidase}}$ cholestenone + H_2O_2

H_2O_2 + 5-aminophenazone + *N*-ethyl-*N*-(3-methylphenyl)-*N*′-succinyl ethylene diamine + H_2O + H^+ peroxidase $\longrightarrow$ quinoneimine dye + H_2O

Absorbance is measured at 600 nm.

D. LDL-CHOLESTEROL

Most of the circulating cholesterol is found in three major lipoprotein fractions: very low density lipoproteins (VLDL), LDL and HDL.

[Total chol] = [VLDL-chol] + [LDL-chol] + [HDL-chol]

LDL-cholesterol is calculated from measured values of total cholesterol, triglycerides and HDL-cholesterol according to the relationship:

[LDL-chol] = [total chol] – [HDL-chol] – [TG]/5

where [TG]/5 is an estimate of VLDL-cholesterol and all values are expressed in mg/dL.

LDL carries most of the circulating cholesterol in man and when elevated contributes to the development of coronary atherosclerosis. LDL-cholesterol is measured to assess risk for CHD and to follow the progress of patients being treated to lower LDL-cholesterol concentrations. Desirable levels of LDL-cholesterol are those below 130 mg/dL in adults and 110 mg/dL in children. In NHANES 1999–2000, LDL-chol will be reported only for fasting participants >5 years of age.

2. SAFETY PRECAUTIONS

A. DAILY SAFETY PRECAUTIONS

All personnel working in the laboratory must wear gloves and laboratory coats. Laboratory coats are to be kept buttoned. Gloves are removed when leaving the immediate work area or when entering offices within the immediate work area. All used gloves, vials, pipettes and other items that come in contact with specimens are disposed of in a Biohazard box lined with a red plastic bag. Such biohazardous waste is picked up each day by housekeeping for proper disposal. Work benches are cleaned at the end of each day with a solution of sodium hypochlorite (bleach:water, 1 :100, v/v) and then covered with plastic-backed white paper.

B. BLOOD HANDLING

The improper handling of blood samples from patients with infectious diseases, e.g., hepatitis or HIV, can lead to infection of staff that draw, handle, analyze, or store such samples. Transmission can occur by ingestion, inhalation, or direct contact, and staff must exercise care when handling blood samples. Always wear liquid impermeable gloves (e.g., latex or plastic) when handling biological samples. Never pipette samples by mouth. Avoid contact with serum. Cover any scratches or cuts on fingers and hands and wear gloves before handling serum. Store all samples in sealed containers. In order to minimize the formation aerosols, do not leave samples open to the atmosphere longer than necessary.

It is about 30 times easier to become infected with hepatitis than with HIV through sample mishandling, and it has been recommended that the usual precautions for handling blood specimens to prevent hepatitis infection serve as a guide to prevent AIDS infection as well. Handle all specimens as if you know them to be infectious. All staff should adhere to the CDC Guidelines for Prevention of HIV Infection in Health Care Workers.

C. SPILLS

The contaminated area is cleaned with a solution of sodium hypochlorite (bleach:water, 1 :100, v/v) and the wipes are disposed of in a red biohazard bag.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

The procedures for data transfer to and from NCHS and WESTAT and within the Laboratory are illustrated in the following figure.

Sample identifying information is received via a closed e-mail system from the NCHS data repository (WESTAT) the same day the samples are received in the laboratory from the MEC. The e-mail sample information is downloaded into a working laboratory database (referred to here as the NHRDR database) and computerized run lists are created for the automated analyzer. When an analytical run is completed, the analytical data are downloaded from the analyzer in the form of ASCII text files, which are then converted to a database and uploaded to the NHRDR database. When all specimen analyses are completed and reviewed, a transmittal file is prepared and sent by e-mail to WESTAT in the form of a comma-delimited file. The quality control data are also transmitted by e-mail at the same time.

The file structures for the electronic shipment log files are described below. The first table indicates general information about the laboratory, address, name of shipment file, and other information. The second table, labeled 'SEND FILE' illustrates the structure of the electronic shipment list transmitted to the laboratory with each batch of specimens. This file is used to import sample information into the laboratory databases.

The third table, labeled "RESULTS FILE: Lipids-Vessel ID 21" shows the format of the transmittal file prepared by the laboratory to transmit results to WESTAT. The first 7 lines in the table contain the information from the "SEND FILE". Note that while the data in the tables on the next two pages break the information down by data field for the sake of clarity, all the data for each NHANES 1999–2000 sample-person is transmitted in a single comma delimited record.

The fourth table, is labeled "QC FILE: Lipid-Vessel ID 21" and illustrates the structure of the quality control transmittal files. Again, all information for each analytical run is transmitted in a single record.

Occasionally, a specimen may have a value considered to be a critical, or “panic value”. A panic value is a value that can reflect a life-threatening problem if not attended to promptly. Of the analytes measured by the NHANES 1999–2000 lipid laboratory, the only one for which such a panic value must be considered is triglyceride. Extremely high triglyceride levels (i.e., levels > 2,000 mg/dL) can result in pancreatitis, a potentially life threatening condition. For this reason, the triglyceride panic value for NHANES 1999–2000 is set at 1,000 mg/dL. Values of 1,000 mg/dL or higher are communicated to NCHS by fax or telephone as soon as they are detected. (See Section 14).

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

A. FASTING

Recent food intake exerts little effect on plasma total cholesterol concentration. Plasma triglycerides, however, increase in postprandial plasma to an extent that is related to the fasting triglyceride levels and the amount of fat intake. This is due to the appearance of chylomicrons in the circulation after a fat-containing meal. Chylomicrons are normally cleared within 9–12 hr, and no chylomicrons should be present after a 12-hr period of fasting. Transient decreases in HDL-chol and LDL-chol also occur, the magnitude of which depends on the fat content of the meal. In NHANES 1999–2000, triglyceride is measured only in specimens drawn from participants who have fasted at least 9 hours before venipuncture.

B. SERUM VS. PLASMA

In general, anticoagulants exert osmotic effects in which water leaves the cells and enters the plasma, thus diluting the plasma and lowering the concentrations of non-diffusible components. The magnitude of this effect depends on the anticoagulant used and its concentration. Serum cholesterol and triglyceride concentrations are about 3–5% higher in serum than in EDTA plasma, although no significant serum-plasma difference was observed for HDL. Thus, the serum concentrations of lipids and lipoproteins probably reflect more accurately the subjects' physiological state at the time of venipuncture. Serum is used for measuring lipids and lipoproteins in NHANES 1999–2000, as it has been for previous HANES surveys.

C. SAMPLE VOLUMES

The sample volumes required are as follows: total cholesterol and/or triglyceride, 0.2 ml; HDL-cholesterol (heparin-Mn^{+2} method), 0.5 ml; HDL measured with the direct method, along with total cholesterol, 0.2 ml. Any sample remaining after analyses are complete are returned to –80°C, and subsequently sent to the NHANES 1999–2000 serum bank as directed by NCHS.

D. SERUM SHIPMENT AND STORAGE CONTAINER.

In NHANES 1999–2000, plastic, screw-top cryovials are used to ship and store serum. Different size vials are used for 3–5 year old children and those >5 years old.

E. STORAGE AND SAMPLE STABILITY

Serum can be stored at –20°C in a non-self-defrosting freezer for up to 4 weeks. For longer storage (> 4 weeks), they should be maintained at –80°C or lower. Total cholesterol, triglyceride, and HDL-cholesterol are stable for at least one year at –80°C or lower.

(1) Collect blood into a glass tube such as a red top Vacutainer blood collection tube.

(2) Allow the blood to stand for 45 min at room temperature to allow complete clotting and clot retraction. A shorter period may result in incomplete clotting and secondary clots may form later. During the clotting period leave the collection tube sealed.

(3) Centrifuge the samples at 1,500 × *g* for 30 min at 4°C. It is preferable to use a refrigerated centrifuge for this purpose, but an unrefrigerated centrifuge can be used if necessary. In either case, the samples should be placed into an ice bath immediately after centrifuging and maintained at 2–4°C thereafter.

(4) Samples should be kept frozen at –20°C, in a non-self-defrosting freezer until shipped to the laboratory. If a shipment must be delayed longer than 4 weeks, the specimens should be kept at –80°C. In the event a shipment may have been thawed and refrozen prior to shipment, this should be noted on the transmittal form.

(5) Samples are shipped by overnight carrier, such as Federal Express. Samples are not shipped on Friday or the day before a holiday, since the laboratory is closed on weekends or holidays. NCHS provided lists of shipment dates that take account of the weekend and holiday schedule. However, in the event it becomes necessary for the laboratory to receive a shipment on a weekend or holiday, NCHS will inform the laboratory of this, and the laboratory makes arrangements to receive the shipment.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this laboratory and procedures specified.

6. PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

Cholesterol, triglyceride and HDL-cholesterol analyses are performed on a Hitachi 704 Analyzer which is serviced by Roche Diagnostics (formerly Boehringer-Mannheim Diagnostics), Indianapolis, IN. Cholesterol is measured enzymatically using the Cholesterol High Performance reagent (cat. no. 704036, Roche Diagnostics). Triglycerides are analyzed enzymatically simultaneously with cholesterol using reagents from the same manufacturer (Triglycerides/GPO, cat. no. 1488872). Triglyceride blanks are measured in CDC surveillance materials using the same reagent, but without lipase. Direct HDL-cholesterol reagent is obtained from Roche Diagnostics (Direct HDL, cat. no. 1661442), and analyzed simultaneously with cholesterol and triglycerides. If for some reason, analyses must be delayed, the specimens should be kept frozen at –80°C until they are analyzed.

Specimens to be analyzed for cholesterol, triglyceride, and HDL-cholesterol can be stored for up to 1 year at –80°C.

A. REAGENTS REQUIRED TO OPERATE INSTRUMENT

(1) Cell Clean 90, cat no. 1224310, Roche Diagnostics. (This is a solution of NaOH, concentration not specified, used to keep reaction cells free of protein deposits). Store at room temperature until expiration date indicated for the lot.

(2) Hitergent, cat no. 409149, Roche Diagnostics. pH 12.5, solution contains 5% ethanolamine, an unidentified antibacterial agent and an unidentified non-ionic detergent. Store at 10–35°C until expiration date indicated for the lot. Working solution, 2% Hitergent. Add 20 mL of Hitergent to deionized water and bring to 1,000 mL. Store at room temperature for up to 4 months.

B. TEST-SPECIFIC REAGENTS

(1) Cholesterol reagent. The components of Cholesterol High Performance System Pack Reagents (Roche Diagnostics, Indianapolis, IN) include (taken from package insert):

Cholesterol reagent (16 x 50 mL):

75 mmol/L PIPES buffer, pH 6.8
10 mmol/L Mg^{2+}
0.2 mmol/L sodium cholate
0.15 mmol/L 4-aminophenazone
> 4.2 mmol/L phenol
> 0.5 U/mL cholesterol esterase (EC 3.1.1.13; *Pseudomonas* species; 25°C)
> 0.15 U/mL cholesterol oxidase (EC 1.1.3.6; *E. coli*; 25°C)
> 0.25 U/mL peroxidase (EC 1.11.1.7; horseradish; 25°C)
1% fatty alcohol-polyglycol ether
Buffer, unspecified stabilizers, unspecified preservative

The reagent is supplied as a solution and is ready to use. After being opened, the reagent is stable for 28 days at 2–12°C, or 7 days at room temperature. Protect reagent from light.

(2) Triglyceride reagents. The components of the Triglycerides (GPO) System Pack include (from package insert):

50 mmol/L PIPES buffer, pH 6.8
40 mmol/L Mg^{2+}
0.20 mmol/L sodium cholate
≥ 1.4 mmol/L ATP
≥ 0.13 mmol/L 4-aminophenazone
4.7 mmol/L 4-chlorophenol
1 μmol/L potassium hexacyanoferrate (II)
0.65% fatty alcohol polyglycolether
≥ 5.0 U/mL lipoprotein lipase (EC 3.1.1.13; *Pseudomonas* species, 25°C)
≥ 0.19 U/mL glycerolkinase (EC 2.7.1.30; *Bacillus stearotheromophilus*; 25°C)
≥ 2.5 U/mL glycerophosphate oxidase (EC 1.1.3.21; *E. coli*; 25°C)
≥ 0.10 U/mL peroxidase (EC 1.11.1.7; horseradish; 25°C)
unspecified preservative

The reagent is supplied as a solution and is ready for use. When opened, the solution is stable for 14 days at 2–12°C, or 7 days at room temperature (15–25°C).

(3) HDL-cholesterol Reagents. Heparin-Mn^{+2} method:

(a) $MnCl_2 \bullet 4H_2O$, 0.62 M. Dissolve 12.269 g of $MnCl_2 \bullet 4H_2O$ in distilled water, transfer quantitatively to a 100-mL volumetric flask, and dilute to volume with distilled water. Store at 4°C.

(b) Heparin, porcine intestinal mucosa, 67.5 mg/mL, 10,000 USP units/mL injectable solution, (Elkins-Sinn, Inc., Cherry Hill, NJ).

(c) Combined reagent. Mix 13.2 ml of heparin solution with 50 mL 0.62 M $MnCl_2$ solution. The precipitant concentrations of the combined reagents are 0.5 M $MnCl_2$ and 14.5 mg/ml heparin. Store at 4°C for up to 3 months.

(d) Sodium bicarbonate, 1.0 M. Dissolve 8.4 g $NaHCO_3$ in deionized water, transfer quantitatively to a 100-mL volumetric flask and dilute to volume with deionized water. Store at room temperature for up to 6 months.

(e) Normal saline (0.9% NaCl). Dissolve 8.775 g NaCl in deionized water. Transfer to a 1,000-mL volumetric flask and dilute to volume with deionized water. (For larger volumes of normal saline, use 17.55 g of NaCl/ 2 L; 35.1 g/4 L. Store at room temperature for up to 3 months.

(4) Equipment

500-µl positive displacement pipettes (SMI, Inc.)

250-µl positive displacement pipettes (SMI, Inc.)

50-µl pipettes, (Absoluter)

1.4-mL microcentrifuge tubes

Wheaton Step-Pette

(5) Direct HDL-cholesterol method

The Direct HDL-cholesterol reagents, R1 and R2, contain the following components (from package insert):

R1 Cyclodextrin/Buffer, supplied as a solution, ready to use.

0.5 mmol/l α-cyclodextrin

0.5 g/L dextran sulfate

7.0 mg/ml magnesium sulfate ($MgSO_4$)

0.3 g/L EMSE

10 mmol/l MOPS (3-morpholino-propane sulfonic acid) buffer, pH 7.0

unspecified preservative

R2 Buffer/PEG-enzyme/4-aminophenazone, is supplied as a lyophilized mixture and is reconstituted with diluent supplied in the reagent kit. R2 contains the following approximate concentrations after reconstitution:

≥ 1 kU/l PEG cholesterol esterase (EC 3.1.1.13; *Pseudomonas* species; 25oC)

≥ 5.6 kU/l PEG cholesterol oxidase (EC 1.1.3.6; *Pseudomonas* species; 25oC)

≥ 30 kU/l peroxidase (EC 1.11.1.7; horseradish; 25oC)

0.5 g/L 4-aminophenazone

10 mmol/L MOPS (3-morpholino-propane sulfonic acid) buffer, pH 7.0

Detergent and preservative (unspecified)

(6) Preparation

The R1 Reagent solution is ready for use. The R2 Reagent (bottle 2a) requires reconstitution using the pre-measured contents of the diluent vial (bottle 2). The procedure is as follows: Carefully pour about 1/2 of the diluent solution (bottle 2) into bottle 2a. Mix gently until the lyophilized material is fully dissolved. Carefully pour the reagent solution back into bottle 2, allowing bottle 2a to drain completely. This transfer should be done as quantitatively as possible, but without rinsing bottle 2a after the transfer. Mix the solution gently for a couple of minutes. The solution is now ready for use. R1 after opening, and R2 after reconstitution are stable for 28 days at 2–12°C.

C. STORAGE AND STABILITY

(1) Cholesterol reagent. Store the open cholesterol System Pack Reagent on the analyzer at 2–12°C. The solution is stable for 4 weeks at 2–12°C or 7 days at 20–25°C when protected from light and contamination by microorganisms.

(2) Triglyceride reagent. Store triglyceride reagent on the analyzer at 2–12°C. The reagent is stable for 14 days at 2–12°C or 7 days at room temperature.

(3) Heparin. Store unopened heparin at 20–25°C. Stable until the expiration date printed on the bottle label.

(4) $MnCl_2$. Store crystalline material in a sealed container at room temperature over $CaCl_2$. Store 0.62 M solution at 4°C. Stable for 2 months.

(5) Combined heparin-$MnCl_2$ Reagent. Store at 4°C. Stable for 2 months.

(6) Direct HDL-cholesterol. R1 Reagent is stable unopened up to the stated expiration date. When open, R1 reagent is stable up to 28 days on the analyzer at 2–12°C. R2 Reagent is stable up to the expiration date prior to reconstitution. After reconstitution, the R2 is stable up to 28 days on the analyzer at 2–12°C.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

The Hitachi analyzer is calibrated at the beginning of the week and as necessary thereafter. A one point calibration procedure is used for total cholesterol, triglyceride and Direct HDL-cholesterol. Frozen serum calibration pools (Soloman Park Laboratories, Kirkland, WA) are used to calibrate total cholesterol and direct method HDL-cholesterol assays. Triglycerides are calibrated using calibration sera (Precical, cat. no 620213) obtained from the Roche Diagnostics. HDL-cholesterol (heparin-Mn^{+2} method) uses a two-point standard curve (standard curve of 0, 50, and 100) and is calibrated using purified cholesterol standards (Preciset, cat no. 125512) obtained from the manufacturer.

A. DAILY CHECK

The following procedures are performed at the beginning of each work day before the first analytical run.

(1) Check water supply.

(2) Check 2% Hitergent supply**.

(3) Check Cell Clean 90 supply**.

(4) Prepare reagents, controls and calibrators as needed.

(5) Exchange incubation bath water: Press MAINTENANCE, then press 1 ENTER.

(6) Perform photometer check.

(7) Air purge: "Start Conditions" display.

(8) Wipe sample and reagent probes.

(9) Probe adjust.

** Refer to Section 6. Reagents, above

B. FULL CALIBRATION

If the CRT display shown is in the Routine Job Menu, press NEXT or BACK to move to the Calibrator & Control Test Selection display. If the CRT display shown is not in the Routine Job Menu, press ROUTINE, then press 3 ENTER.

(1) PHASE: The Instrument stores operating parameters and calibration data for two (2) sets of twenty chemistries each. These two sets of chemistries are referred to as PHASE 1 and PHASE 2. The two phases are defined in the Channel Assignment display. To indicate which set of chemistries is being used, type the phase number (1 or 2), the press ENTER. For NHANES 1999–2000, all tests are in the PHASE 1 set.

(2) CALIBRATION TYPE: Press 1 ENTER to specify "Start-Up" Calibration.

(3) STANDARD TYPE: Press 1 ENTER to select tests for the blank (saline). If test selection for the blank is stored in memory, the tests in memory appear at the right margin of the display.

(4) TESTS: Activate the appropriate test or profile keys for those tests which require a Blank Calibrator, then press ENTER. (Each test key is activated when its LED is illuminated). The tests assigned appear at the right margin of the display, and the STANDARD TYPE entry field displays: "STD 2-6".

(5) TESTS: Press the appropriate test keys for those tests which require a standard or standards, then press ENTER. The tests assigned appear at the right margin of the display and the STANDARD TYPE entry field displays: "ISE 1,2". Advance the cursor to the CALIB LOAD LIST entry field. Press 1 ENTER.

(6) If you do not want to run controls, update the System Disk with calibrator test selection as follows:

Advance the cursor to FD READ/WRITE.
Press 2 ENTER.
The CRT displays: "WRITE OK?"
Press 1 ENTER (YES).

NOTE: It is not absolutely necessary to write calibrator test selection data on the System Disk. However, if the laboratory experiences a power failure, this step prevents permanent loss of test selection information. Wait while the System Disk is updated, then proceed to ROUTINE PATIENT TEST SELECTION (Section 2.1.6).

C. BLANK CALIBRATION ONLY

If the CRT display shown is in the Routine Job Menu, press NEXT or BACK to move to the Calibrator & Control Test Selection display. If the CRT display shown is not in the Routine Job Menu, press ROUTINE, then press 3 ENTER.

(1) PHASES: The instrument stores operating parameters and calibration data for two (2) sets of twenty chemistries each. These two sets of chemistries are referred to as PHASE 1 and PHASE 2. The two phases are defined in the Channel Assignment display. For NHANES 1999–2000, all tests are in the PHASE 1 set.

To indicate which set of chemistries is being used, type the phase number (1 or 2), then press ENTER.

(2) CALIBRATION TYPE: Press 1 ENTER to specify "Start-Up" Calibration.

(3) STANDARD TYPE: Press 1 ENTER to select tests for the blank (saline) update. If previous test selections for blanks are stored in memory, the tests in memory will appear at the right margin of the display.

(4) TESTS: Activate the appropriate test or profile keys for those tests requiring a blank update, then press ENTER. (Each test key is activated when its LED is illuminated.) The tests assigned appear at the right margin of the display, and the STANDARD TYPE entry field displays: "STD 2-6".

(5) TESTS: Deselect all previously selected tests so that no tests are selected for "STD 2-6", then press ENTER. No tests should appear at the right margin of the display, and the STANDARD TYPE entry field now displays: "ISE 1,2".

(6) Press ROUTINE, then press 4 ENTER and the Start Conditions screen will appear on the display.

(7) Enter the START SAMPLE NO. and request START UP CALIBRATION. Verify that a control interval of 1 or greater has been selected. All runs of 15 samples or more require 3 sets of control pools per run. (Tests requiring controls were selected in Routine Job No. 3)

(8) Press START to begin the calibration.

D. REAGENT/CALIBRATOR CHANGES

The primary means of following any manufacturer initiated changes in reagent or calibrator formulations is the laboratory quality control system. Such reagent or calibrator changes are generally minor, if visible at all. However, they have the potential for introducing minor abrupt shifts in the laboratory mean, which are normally detected through the QC system.

When the laboratory is informed of an impending change, the Laboratory Operations Coordinator will take steps to procure the new reagent or calibrator in sufficient time to perform analyses in parallel with the old and new reagent or calibrator. Such parallel analyses will be conducted over a period of 3-4 weeks in 10 runs, each of which is performed on a different day. Each parallel run will be performed on the same day, and will include the appropriate QC pools and at least 20 specimens, for a total of 200 specimens over the 10 runs. This is accomplished by setting up additional instrument channels with the new reagent or calibrator and analyzing the sample simultaneously in both the normal and new channels.

At the end of the 10 runs, descriptive statistics [mean (SD), %CV] will be determined for each analyte in each QC pool, and a paired t-test will be used to assess the significance of the differences between like analytes in each pool. Descriptive statistics (mean; SD), paired t-tests, and linear regression analyses relating the two arms of the parallel analyses will also be conducted for the affected analytes in the 200 split specimens analyzed with the old and new reagents or calibrators. These data will serve to characterize the effect of new reagent or calibrator formulations on NHANES 1999–2000 analyses.

Note that the laboratory has no control over reagent or calibrator formulations, and will have to use the new reagent if it replaces the current formulation. The data collected above, however, will be useful during the data analysis phase when the desirability of adjusting NHANES 1999–2000 data to account for such systematic bias changes can be considered. Based on past experience, such systematic biases are expected to be minimal and would probably be considered acceptable without adjustment. Nonetheless, the data will be available for such adjustment should this not be the case.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

The assay conditions for each test are defined by the Instrument Settings for that test. The instrument settings are provided by the manufacturer. They define the time, temperature, reagent and sample volumes, calibration parameters and other information required for the instrument to analyze the specimens and calculate the results. The instrument settings for NHANES 1999–2000 measurements are as follows.

A. INSTRUMENT SETTINGS

(1) Cholesterol

Temperature: 37°C

Test [CHOL]

Assay Code	[1POINT]:[15]–[0]
Sample Volume	[4]
R1 Volume	[350][50][NO]
R2 Volume	[0][20][NO]
Wavelength	[700][505]
Calib. method	[LINEAR][0][0]
Std. (1) Conc.-Pos.	[0]–[1]
Std. (2) Conc.-Pos.	[325]–[5]
Std. (3) Conc.-Pos.	[0]-[0]
Std. (4) Conc.-Pos.	[0]–[0]
Std. (5) Conc.-Pos.	[0]–[0]
Std. (6) Conc.-Pos.	[0]–[0]
Unit	[MG/DL]
SD Limit	[0.1]
Duplicate Limit	[200]
Sensitivity Limit	[1500]
ABS. Limit (INC/DEC)	[0][INC.]
Prozone Limit	[0][LOWER]
Expected Value	[0]–[250]
Instrument Factor	[1.00]

(2) Triglyceride

Temperature: 37°C

Test	[TRIG]
Assay Code	[1POINT]:[32]–[0]
Sample Volume	[3]
R1 Volume	[350][50][NO]
R2 Volume	[0][20][NO]
Wavelength	[700][505]
Calib. Method	[LINEAR][0]
Std. (1) Conc.-Pos.	[0]–[1]
Std. (2) Conc.-Pos.	[137]–[2]
Std. (3) Conc.-Pos.	[0]–[0]
Std. (4) Conc.-Pos.	[0]–[0]
Std. (5) Conc.-Pos.	[0]–[0]
Std. (6) Conc.-Pos.	[0]–[0]
Unit	[MG/DL]
SD Limit	[0.1]
Duplicate Limit	[200]
Sensitivity Limit	[1100]
ABS. Limit (INC/DEC)	[0][INC.]
Prozone Limit	[0][LOWER]

Expected Value	[0]–[200]
Instrument Factor	[1.00]

(3) HDL-cholesterol (heparin-Mn^{+2} supernatants)

Temperature: 37°C

Test	[HDL]
Assay Code	[1POINT]:[15]–[0]
Sample volume	[5]
R1 Volume	[500][50][NO]
R2 Volume	[0][50][NO]
Wavelength	[700][505]
Calib. Method	[NONLINEAR][4][3]
Std. (1) Conc.-Pos.	[0]–[1]
Std. (2) Conc.-Pos.	[50]–[2]
Std. (3) Conc.-Pos.	[100]–[3]
Std. (4) Conc.-Pos.	[0]–[0]
Std. (5) Conc.-Pos.	[0]–[0]
Std. (6) Conc.-Pos.	[0]–[0]
Unit	[MG/DL]
SD Limit	[0.1]
Duplicate Limit	[100]
Sensitivity Limit	[700]
ABS. Limit (INC/DEC)	[INC.]
Prozone Limit	[0][LOWER]
Expected Value	[25]–[85]
Instrument Factor	[0.21]

(4) Direct HDL-cholesterol

Temperature: 37°C

Test	[DHDL]
Assay Code	[2POINT]:[15]–[32]
Sample volume	[4]
R1 Volume	[300][50][NO]
R2 Volume	[100][20][NO]
Wavelength	[700][600]
Calib. Method	[LINEAR][0][0]
Std. (1) Conc.-Pos.	[0]–[1]
Std. (2) Conc.-Pos.	[51.4]–[2]
Std. (3) Conc.-Pos.	[0]–[0]
Std. (4) Conc.-Pos.	[0]–[0]
Std. (5) Conc.-Pos.	[0]–[0]

Std. (6) Conc.-Pos.	[0]–[0]
Unit	[MG/DL]
SD Limit	[0.1]
Duplicate Limit	[100]
Sensitivity Limit	[700]
ABS. Limit (INC/DEC)	[2000][INC.]
Prozone Limit	[32000][UPPER]
Expected Value	[35]–[65]
Instrument Factor	[.00]

B. CALCULATION

The Hitachi 704 microcomputer uses absorbance measurements to calculate cholesterol, triglyceride, and HDL concentrations as follows:

$$C_x = [K(A_x - A_b) + C_b] \times IF$$

Where:

Cx = Concentration of sample.

K = Concentration factor (determined during calibration).

Ax = Mean of absorbances of Sample + R1 read during cycles indicated in the Assay Code field for the respective test.

Ab = Mean of absorbances of Blank +R1 read during cycles indicated in the Assay Code field for the respective test.

Cb = Concentration of Blank (STD).

IF = Instrument Factor (dilution correction). A factor of 1.21 is used for HDL-chol by the heparin-Mn^{+2} method to account for sample dilution upon adding the precipitation reagents. IF = 1.00 for cholesterol, triglyceride, and direct method HDL-cholesterol.

C. SAMPLE ANALYSIS PROCEDURES

(1) Receipt of samples in laboratory. When the samples arrive in the laboratory, they are logged into the laboratory by batch according to the shipping transmittal that accompanies the samples. The laboratory records the date the samples were received and their condition (O.K., thawed, sample missing, etc.) using the appropriate sample condition code (Appendix A). The samples are transferred to a –80°C freezer until they are analyzed.

(2) Inadequate specimens. Inadequate specimens can result from factors such as cracked vials, inadequately sealed vials, empty vials, gross hemolysis, and thawed samples. When they occur occasionally, such inadequate specimens are noted on the transmittal using the sample condition codes. If the problem involves an entire shipment, or reflects a continuing problem, the originating MEC will be alerted by fax as soon as the Laboratory becomes aware of the problem.

(3) Preparation of samples for analyses. The vials are removed from the freezer, placed upright and allowed to thaw at room temperature. The sealed vials are placed on a blood mixer and rotated for 30 min at room temperature to ensure complete mixing. The samples are then unsealed and aliquots are removed for the appropriate tests. After thawing, specimens are stored at 4°C. The schedule sample receipt, analysis, data checks and preparation of transmittal files is indicated in section 6. above. Analyses should be performed within the first 2 days after thawing and repeat analysis should be performed within 6 days.

(4) Interferences. Hemolysis can interfere with absorbance readings. Lipemia can affect the triglyceride measurements by interfering with absorbance measurement. Grossly turbid samples

are diluted before analysis. An aliquot of the specimen is diluted with normal saline to an extent sufficient so that the value measured in the diluted specimen remains within the range 1–1,000 mg/dL. Depending on the triglyceride concentration, sample dilutions will generally be within the range from 1:2 (i.e., 1 part specimen + 1 part saline, v/v) to 1:20 (i.e., 1 part specimen + 19 parts saline, v/v). In addition, samples with triglyceride values exceeding 400 mg/dL and cholesterol values exceeding 400 mg/dL are reanalyzed to confirm the high values.

(5) Sample analysis. The Hitachi analyzer is programmed by downloading the specimen ID numbers and test codes (for total cholesterol, triglyceride, HDL-cholesterol and direct HDL-cholesterol, as appropriate) in machine readable form. The download file is a database prepared from the electronic sample shipment log. Cholesterol, triglycerides and direct HDL-cholesterol are analyzed simultaneously. Heparin-Mn^{+2} supernatants are analyzed in a separate run. For each sample, the instrument performs the analyses indicated by the test codes for that sample.

(6) Preparation of heparin-Mn^{+2} supernatants.

(a) allow samples and quality control sera (LR and SL4 pools, one each per batch of 40 samples) and 0.5 M MnCl2/heparin combined reagent to come to room temperature. The quality control samples are carried through the same procedure as the actual samples.

(b) Number a sufficient quantity of microcentrifuge tubes, two tubes per specimen.

(c) Transfer 0.50 ml of sample to the first microfuge tube. Rinse the pipette with saline solution t three times before pipetting the next sample.

(d) Add 50 µl of combined reagent. Mix by vortexing three times for 5 sec. Allow to stand at room temperature for 15 minutes.

(e) Sediment the precipitate by centrifuging at 10,000 × *g* for 9 minutes.

(f) Carefully remove the tubes from the centrifuge. Do not disturb the precipitate.

(g) Transfer 250 µl of the supernatant to the second microcentrifuge tube. Add 25 µl of 1 M NaHCO3. Mix by vortexing three times for 5 sec. Let stand for 10 minutes at room temperature. (At this point the samples are stable and if necessary, can be stored overnight before analysis.)

(h) Centrifuge at 10,000 × *g* for 3 min minutes to sediment the MnCO3 formed.

(i) Analyze the supernatant for HDL-cholesterol. Analyze the quality control samples precipitated with the batch, using selecting the heparin-Mn+2 HDL channel of the Hitachi Analyzer. This channel is programmed to multiply the observed value by the factor 1.21 to correct for dilution of the sample by the reagents.

NOTES

- Samples of low volume, and those which are extremely turbid, are diluted two fold. Dilute 250 µl of sample with 250 µl of normal saline. Mark tubes containing diluted samples “1→2” and place in a yellow Eppendorf microcentrifuge tube at the final step. The convention adopted by the Lipoprotein Laboratory is to refer to a sample as having been diluted 1:2 if the original concentration has been reduced by half. A 1:3 dilution reduces the concentration three-fold, and would contain 1 vol of sample plus 2 vols of normal saline.
- Hitachi channels - Serum HDL-chol is analyzed on Hitachi channel 13.
- Control sera are also analyzed in the serum HDL channel.

(7) Analysis of lipids and lipoprotein cholesterol. The reaction conditions are established by the Instrument Settings (see Section 8. Procedures, above). Place a 100-µl aliquot of sample into the disposable sample cups on the instrument carousel using a disposable polyethylene transfer pipette. Arrange the samples on the carousel in the order in which they are to be analyzed, as determined from the download file (see Section 10 e, above). Place the quality control samples

into their assigned positions on the instrument and begin the analysis. The results are printed on the Hitachi printout and also sent in real time to computer text files on a dedicated computer. A separate file is created for each sample. These are assembled into a single database at the end of the day.

(8) Expected Values.

The expected values for cholesterol, triglyceride, and HDL-cholesterol are as follows:

Cholesterol Conc (mg/dL)	Interpretation
< 200	Desirable
200–239	Borderline-High
> 240	High

Triglyceride Conc (mg/dL)	Interpretation
< 200	Desirable
200–400	Borderline
400–1,000	High
>1,000	Very High

HDL-cholesterol (mg/dL)	Interpretation
< 35	Low
>60	High

(9) Quality control. The quality control results are copied from the Hitachi run printout onto the Run cover sheet, and examined by the technician. If the results exceed control limits, the source of the difficulty is identified and corrected, and the run is repeated. Specimen results falling outside of expected ranges defined for each analyte (see section 9), are confirmed. When it is necessary to confirm a high triglyceride value, the specimen is diluted as appropriate for the repeat analysis. When a sample is very turbid and it is expected that the value will be off scale, the sample is diluted for the initial analysis. In extremely turbid samples (milky in appearance), it may be necessary to use a dilution of 1:4 or higher.

(10) Analysis review. After analysis and review of the quality control measurements, the results from 'in-control' runs are downloaded to the NHANES working database (NHRDR database). When all analyses are complete, they are reviewed by the Laboratory Technician (Preliminary Review) to ensure that all necessary repeat analyses have been performed. The results are then reviewed by the Laboratory/Study Coordinator (Final Review), using a series of visual and computer checks for completeness, consistency of the measurement, and quality control status.

(11) Repeat ranges. The results are reviewed using ranges based on the normal population distribution determined in NHANES III. The purpose is to help detect analytical errors, and when values fall outside defined ranges, they are considered to be unlikely values and are confirmed. The ranges we use for total cholesterol, triglyceride and HDL-cholesterol are as shown below.

	Age < 12 years	Age >12 years
cholesterol	100-300 mg/dL	140-400 mg/dL
triglyceride	25-400* mg/dL	25-400* mg/dL
HDL-Cholesterol	20-85 mg/dL	20-85 mg/dL

* If the sample is turbid, it will generally have a high triglyceride. In this case the upper limit for triglyceride is 600 mg/dl; Values above this level are confirmed by repeating the analysis after appropriately diluting the sample.

(12) Reportable range of test results

The expected values for cholesterol, triglyceride, and HDL-cholesterol are as follows:

Cholesterol Conc (mg/dL)	Interpretation
< 200	Desirable
200-239	Borderline-High
> 240	High

Triglyceride Conc (mg/dL)	Interpretation
< 200	Desirable
200-400	Borderline
400-1,000	High
>1,000	Very High

HDL-cholesterol (mg/dL)	Interpretation
< 35	Low
>60	High

(13) Quality control (QC) procedures

The Central Laboratory monitors its performance by analyzing quality control sera for which the values have been assigned by the Centers for Disease Control (CDC) Lipid Standardization Laboratory using CDC reference methods. The estimates of analytical error obtained from the analysis of quality control materials are assumed to represent the error of the measurements in survey samples. The control pools are therefore subjected to the same analytical manipulations as the survey samples.

The precision of lipid and lipoprotein analyses is determined from replicate analyses of the control sera in each run. Two control pools, one with normal and one with elevated lipid concentration, are used to monitor the analysis of total plasma cholesterol and triglyceride. Similarly, two levels of control sera are used for HDL-cholesterol, one at the level of about 35 mg/dL and the other at about 50 mg/dL.

(a) Control limits. The control limits for each pool are calculated from the overall mean and standard deviation of the run means, and ranges for the pool. Temporary control limits for each pool are calculated from the first 20 run days. Permanent control limits are determined after 50 run days and remain in effect until the pool is exhausted. Continuity between the current and replacement pool is maintained from at least 20 overlapping runs in which both pools are analyzed in parallel. It is from this period of overlap that the 20 run temporary limits are established for the replacement pool. During this period the acceptability of the measurements is based on the current pool. Furthermore, the analyses must be "in control" before the data are accepted for use to establish control limits for the replacement pool. Two types of control charts are prepared for each level of each analyte. The mean chart monitors the deviation of individual run means X from the overall laboratory mean, X. Any shift, drift, or among day variability is assessed from the mean chart. The range, or R chart, monitors within-run variability.

(b) Quality Control Pools. Two quality control pools are used to monitor the analysis of total cholesterol and triglyceride. Two other pools are used for HDL-chol. In each case, one pool

has normal, and the other elevated concentrations of the respective analytes. An aliquot from each pool is analyzed three times in each run.

(c) Introduction of Replacement Control Pools. Before a control pool is depleted, a replacement pool is purchased from Solomon Park Laboratories, Kirkland, WA. These pools have CDC-assigned reference values. Each is analyzed on a minimum of 20 run days (temporary limits) concurrently with the current pool. The mean, standard deviation, and range for the replacement pool are established. During this overlap period, quality control is maintained with the current pool.

Limits for the replacement pool are calculated and evaluated, and control charts are prepared as described in the following sections. Care is taken to assure that data used in the calculations are only from runs that are "in control" i.e. that meet established quality control criteria. As soon as acceptable temporary limits are reestablished, control is transferred to the replacement pool, and the original pool is retired. Permanent control limits are established after 50 run days.

The Laboratory is directly standardized for cholesterol, triglyceride and HDL-cholesterol measurements through the CDC-NHLBI Lipid Standardization Program. As part of this program, the laboratory undergoes continuous external surveillance by CDC to maintain standardization. CDC Standardization samples are normally received quarterly. Each shipment contains 36 specimens, identified for analysis in four analytical runs of 9 samples each. Each sample is analyzed in duplicate. Thus, the four standardization runs are analyzed over a 12 week period, or one run each three weeks.

Standardization is assessed for each quarter by CDC. This assessment is based on the results from all four runs, which must be completed before the assessment can be made. Our primary aim is that the Lipid Standardization measurements are distributed more or less equally throughout the entire year, rather than each quarter’s samples being analyzed during one short period during the quarter. In this way, the data more nearly reflects continuous, rather than periodic laboratory performance. For this reason, one CDC standardization run will be made every three weeks (+1 week to account for unforeseen delays). The data will be transmitted to CDC and evaluated upon completion of all four runs.

The following table summarizes Laboratory quality control performance at the beginning of the NHANES 1999–2000 survey.

Quality control results for period 07/20/98 to 09/17/98

Analyte	Pool	CDC ref value	*n*	Lab mean (SD)	% CV	% Bias
tot chol	SL2I080	177.2	36	175.8 (1.6)	0.9%	-0.8%
	SL3I080	251.1	36	250.3 (2.7)	1.1%	-0.3%
trig	SL2I080	98.6	35	95.6 (2.5)	2.6%	-3.2%
	SL3I080	215.3	35	213.8 (3.3)	1.6%	-0.7%
HDL (hp-Mn^{+2})	LR9806	35	12	35.1 (1.4)	4.0%	0.4 %
	SL4I080	49.8	12	50.4 (1.6)	3.2%	1.1%
HDL (direct)	LR9806	35	29	33.6 (1.0)	2.9%	–3.9%
	SL4I080	49.8	30	49.6 (1.0)	2.1%	–0.3%

(d) Calculation of Control Limits. The Lipid Laboratory uses statistical control charts to evaluate performance and make quality control decisions. Control limits are calculated from the means, standard deviations and ranges as described in this section. It is important that the data used to calculate control limits be collected during a stable analytical period when they are representative of overall laboratory performance. The daily mean, X, for a control pool is calculated for each run by averaging the replicate values for the pool:

X = sum of control values/number of replicates = x/*n*

For NHANES 1999–2000, *n* = 3.

The overall mean for the pool, X, is calculated by summing the individual run means and dividing by the number of runs, *N*:

X = sum of run means/number of runs = X/N

The overall mean is rounded to the nearest whole number.

N = 20 run days for temporary limits

N = 50 run days for permanent limits

The standard deviation of the run means, S_X, is also calculated for the control pool. The basic equation for calculating standard deviation is as follows:

$S_X = (x - x)^2/(N - 1)$

The range, R, for each run is the difference between the highest and the lowest value obtained for the pool in that run:

$R = X_{high} - X_{low}$

The average range, R, for a series of runs is calculated by dividing the sum of the ranges for the series by the number of runs:

R = R/*N*

N = 20 for temporary limits

N = 50 for permanent limits.

The control limits (99%) for the X chart are calculated as follows:

Upper control limit = $X + 3S_X$

Lower control limit = $X – 3S_X$

Control limits are rounded to the nearest whole number.

The warning (95%) limits for the X chart are calculated as follows:

Upper warning limit = $X + 2S_X$

Lower warning limit = $X – 2S_X$

Warning limits are rounded to nearest whole number.

The limits on X are evaluated as described below.

The limits used for the R chart are calculated in a similar fashion.

Range control limit = $R + 3\ S_r$

Range warning limit = $R + 2\ S_r$

where S_r is the standard deviation of R.

The lower limit for the range chart is zero since there is no negative range.

(e) Evaluation of Control Limits. Before the control chart can be used for quality control, it is reviewed to determine that the data have been collected during a stable analytical period. The chart is examined for outliers, for periods of questionable or unstable performance, and for evidence of excessive bias. An outlier will distort the control limits if incorporated into the final calculations. An outlier is considered to be any value of X which falls outside the control limits (X + 3Sx) or any value of R which exceeds the control limit for R. These values are eliminated as are values from any questionable period of performance. The values of X, Sx, and the control limits are recalculated and the charts are evaluated again.

When values from at least 20 acceptable runs are used for the final calculations, the control charts are constructed according to the criteria listed below. If there are not 20 acceptable runs after eliminating unacceptable data, continue analyzing the pool until at least 20 acceptable runs have been completed.

The criteria used in the Lipid Laboratory were those that served as guidelines for the Lipid Research Clinics Program and are designed to minimize both bias and variability. As used in this manual, the bias of the cholesterol, triglyceride or HDL-cholesterol measurement is calculated as the algebraic difference between the X and the CDC reference value (RV) for the pool.

(f) Construction of Control Charts. A separate control chart is constructed for each analyte in each control pool. Construct each chart so that plots for X and R are arranged one above the other on the same sheet of graph paper. Draw the X line across the entire sheet; draw the warning- and control limits parallel to the X line. At the top of the chart, indicate the CDC reference value. Draw the R line and R limits on the R plot.

Plot the run mean and range values. The chart should be kept current; the values should be plotted after each run. Make liberal use of annotations indicating events that might affect the analyses (personnel changes, reagent problems, changes in instrument components, etc.).

(g) Use and Interpretation of Control Charts. Values for X which exceed the $3S_X$ limit or values of R that exceed the range control limit indicate the run is ‘out-of-control’. The run must be

repeated. Statistically, one in 100 runs can be expected to be 'out-of-control during normal stable operation. A value exceeding the warning limit, but not the control limit, is interpreted as an indication of possible trouble, but does not necessarily require action. Statistically, about one in 20 values will exceed the warning limits.

(h) Actions taken when analyses are not acceptable. In cases where a single control pool falls outside specified ranges, but calibration is acceptable and the other control pool is acceptable, a decision may be made to repeat 10% of the samples from the technically out of Control run, and if these values are confirmed in an in control run, the run may be accepted. This decision is made by either the Lab Director or the Laboratory/Study Coordinator.

When runs are consistently out of control, the calibrators, reagents and other material are checked to make sure they are not out of date. The Hitachi 704 troubleshooting guide is consulted and calibration is repeated.

Replacement control pools are analyzed to obtain temporary limits (20 run days). Final limits are calculated after 50 run days. A new QC graph is prepared each time a pool lot changes and is recreated when limited are created, temporary or permanent.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the run is declared "out of control", the system (instrument, calibration standards, etc.) is investigated to determine the root of the problem before any results are released. Consult with the supervisor for appropriate actions.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

A. CHOLESTEROL AND TRIGLYCERIDE LIMITATIONS

The following information applies to both cholesterol and triglyceride.

Hemolysis:	No interference up to 200 mg/dL hemoglobin.
Lipemia:	No interference unless lipemia is marked, generally over 1,000 mg/dL. Turbid specimens are automatically flagged by the analyzer, and are reanalyzed after dilution with 0.15 M NaCl. Note that in NHANES 1999–2000, specimens with TG > 600 mg/dL are diluted with 0.15 M NaCl and reanalyzed.
Bilirubin:	No interference up to 12 mg/dL.

The color reactions used to measure cholesterol and triglyceride (Section 1 a. and b., above) are unaffected by common interfering substances such as uric acid, creatinine, and glutathione.

Drug Interference.

The following information was taken from package insert. No further details were provided by the manufacturer:
Therapeutic amounts of 38 drugs showed no interference with this procedure.

A two-fold toxic dose of α-methyldopa lowered recovery by 50%.

Noramidopyrine showed a 20% lowered recovery.

A ten-fold therapeutic concentration of ascorbic acid led to 5% lower recovery of cholesterol.

B. HDL-CHOLESTEROL LIMITATIONS

(1) Heparin-Mn^{+2} precipitation. If the supernatant is not clear, complete precipitation of LDL and VLDL has not occurred and will result in falsely elevated HDL-cholesterol values. Samples from patients with certain hyperlipidemias resulting in extremely high triglyceride concentrations, and dysproteinemias may not be precipitated adequately. Such inadequate precipitation is evident

from the a) turbidity of the supernatant after centrifugation; or b) appearance of part of the turbidity as a lipid rich floating layer. Such supernatant are not analyzed. If there is sufficient specimen volume to repeat the precipitation, the specimen is diluted with an equal volume of 0.15 M NaCl, and the heparin-Mn^{+2} precipitation is repeated. If the resulting supernatant is clear, the specimen is analyzed. If there is insufficient volume to repeat the heparin-Mn^{+2} precipitation, or if the second supernatant is also inadequate, the specimen is not analyzed for HDL-cholesterol, and the value is reported as missing.

Possible interferences with cholesterol measurement are indicated above.

(2) Direct HDL-cholesterol limitations (from package insert): Icterus: No significant* interference from unconjugated bilirubin up to an icteric index of 65 or from conjugated bilirubin up to an I index of 18.

Hemolysis: No significant* interference from hemoglobin up to an hemoglobin index of 1000.

Lipemia: No significant* interference from triglycerides up to 1,200 mg/dl.

Ascorbic Acid: No significant* interference from ascorbic acid up to 15 mg/dl.

* the term 'significant' was not defined by the manufacturer.

13. REFERENCE RANGES (NORMAL VALUES)

The reference ranges are the population distributions for cholesterol, triglyceride and HDL-cholesterol, as determined in NHANES III. These are shown in Appendix B. These distributions were used to create the automatic repeat ranges in Section 10.k.

14. CRITICAL CALL RESULTS (PANIC VALUES)

triglyceride > 1,000 mg/dL
cholesterol > 400 mg/dL

There is no critical call range for HDL-cholesterol.

Very high triglyceride values can lead to a medical emergency. Therefore, whenever Critical Call Results are obtained for triglyceride, the sample is repeated immediately. Upon confirmation of the results, the information is given to NCHS by telephone or FAX. Faxes are generally used because the FAX and its confirmation message serve as documentation for the laboratory and NCHS.

No immediate medical emergency is associated with a cholesterol value above 400 mg/dL. NCHS has requested similar notification of cholesterol values > 400 mg/dL, however, and the notification procedures used for triglyceride are also used for cholesterol.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens should be maintained at 20–25°C during testing. After testing, the samples are stored at –70°C or colder.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

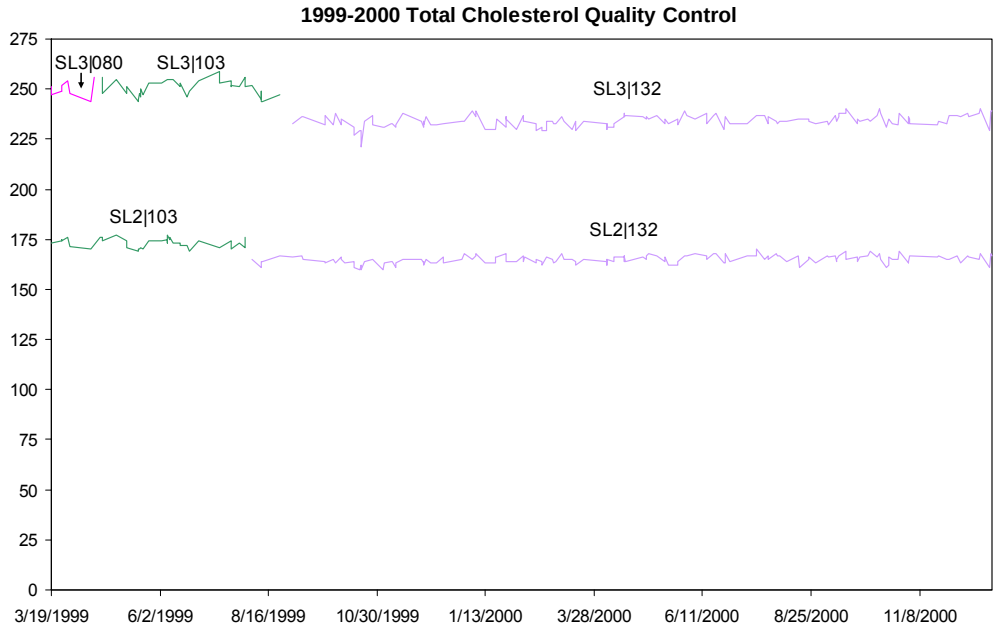
There are no acceptable alternative methods of analysis. Otherwise, specimens should be stored at –70°C or colder until the system is returned to functionality.

17. SUMMARY STATISTICS AND QC GRAPHS

A. TOTAL CHOLESTEROL

Summary Statistics for Total Cholesterol by Lot

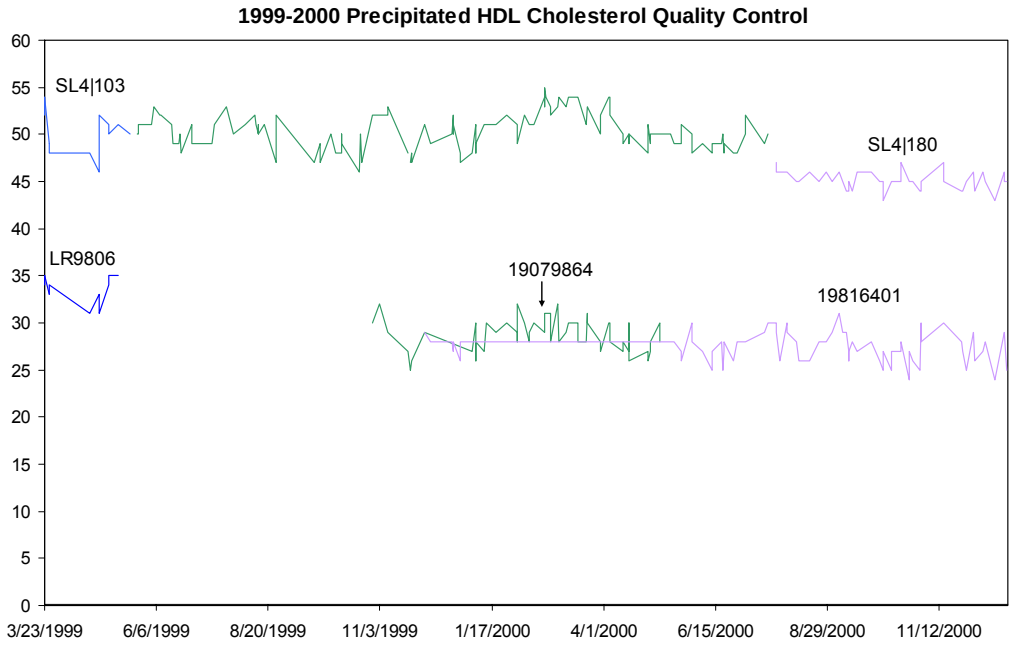
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SL3I080	9	3/19/1999	4/22/1999	250.2	3.7	1.5
SL2I103	43	3/19/1999	7/31/1999	173.0	2.2	1.3
SL3I103	40	4/23/1999	8/24/1999	251.1	4.0	1.6
SL2I132	167	8/5/1999	12/28/2000	165.0	2.1	1.2
SL3I132	161	9/2/1999	12/28/2000	234.1	2.9	1.2



B. PRECIPITATED HDL CHOLESTEROL

Summary Statistics for HDL Cholesterol by Lot

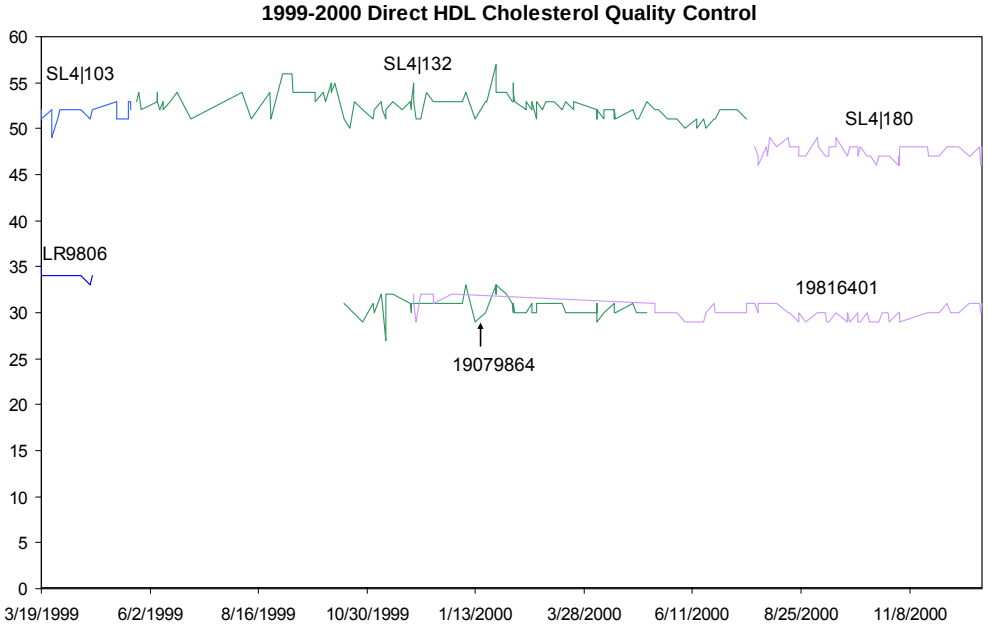
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SL4I103	12	3/23/1999	5/19/1999	49.9	2.2	4.5
LR9806	10	3/23/1999	5/11/1999	33.5	1.5	4.5
SL4I132	132	5/20/1999	7/20/2000	50.3	1.9	3.8
19079864	60	10/29/1999	5/9/2000	28.6	1.8	6.2
19816401	78	11/3/1999	12/27/2000	27.5	1.6	5.7
SL4I180	49	7/26/2000	12/27/2000	45.1	0.9	2.1



C. DIRECT HDL CHOLESTEROL

Summary Statistics for Direct HDL Cholesterol by Lot

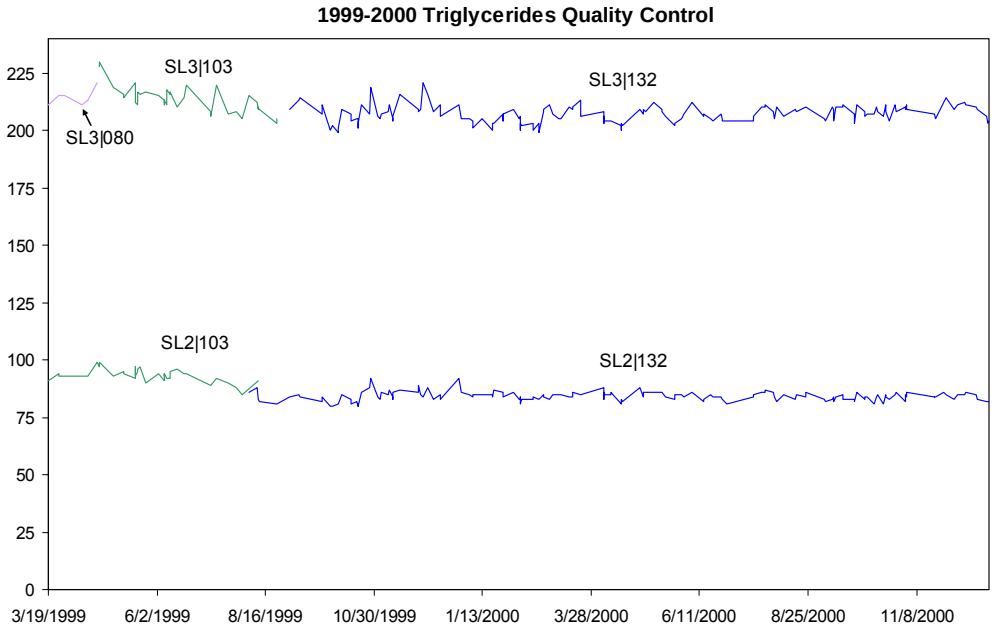
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LR9806	8	3/19/1999	4/23/1999	34.0	0.5	1.6
SL4I103	17	3/19/1999	5/20/1999	51.8	1.0	2.0
SL4I132	124	5/21/1999	7/18/2000	52.5	1.4	2.6
19079864	60	10/14/1999	5/10/2000	30.6	1.1	3.5
19816401	85	11/12/1999	12/28/2000	30.1	0.9	3.0
SL4I180	53	7/24/2000	12/28/2000	47.4	0.8	1.6



D. TRIGLYCERIDES

Summary Statistics for Triglycerides by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SL2I103	39	3/19/1999	8/11/1999	93.1	2.9	3.1
SL3I080	8	3/19/1999	4/22/1999	214.1	3.3	1.5
SL3I103	37	4/23/1999	8/24/1999	213.5	5.9	2.8
SL2I132	163	8/5/1999	12/28/2000	84.2	2.0	2.4
SL3I132	157	9/2/1999	12/28/2000	206.9	3.8	1.8



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