Laboratory Procedure Manual

Analyte: **Total Cholesterol**

Matrix: **Serum**

Method: **Hitachi 717**

as performed by: *Lipid Laboratory Johns Hopkins*

University School of Medicine

Lipoprotein Analytical Laboratory

600 North Wolfe Street
Blalock 1379
Baltimore, MD 21287
410-614-1030

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November 2007

Important Information for Users

The Johns Hopkins Lipid Laboratory 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 testing the items listed in the following table:

File Name	Variable Name	SAS Label
TCHOL_d	LBXTC	Total cholesterol (mg/dL)
	LBDTCSI	Total cholesterol (mmol/L)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Cholesterol is measured enzymatically in serum 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:

Sample, addition of R1 (cholesterol reagent) and start of reaction: Cholesterol is determined enzymatically using cholesterol esterase and cholesterol oxidase.

$$\text{cholesterol esters} + H2O \xrightarrow[\text{esterase}]{\text{cholesterol}} \text{cholesterol} + \text{RCOOH}$$

Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids.

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

Cholesterol is converted by oxygen with the aid of cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide.

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

Hydrogen peroxide created forms a red dye by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase. The color intensity is directly proportional to the concentration of cholesterol and can be determined photometrically.

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.

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 snapped. Lab coats must meet OSHA compliance CPL2-2.44D. Splash and spray resistant fabric that is also antistatic is required. 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. Work benches are cleaned at the end of each day with a solution of sodium hypochlorite (bleach: water, 10:100, v/v) and then covered with plastic-backed white paper.

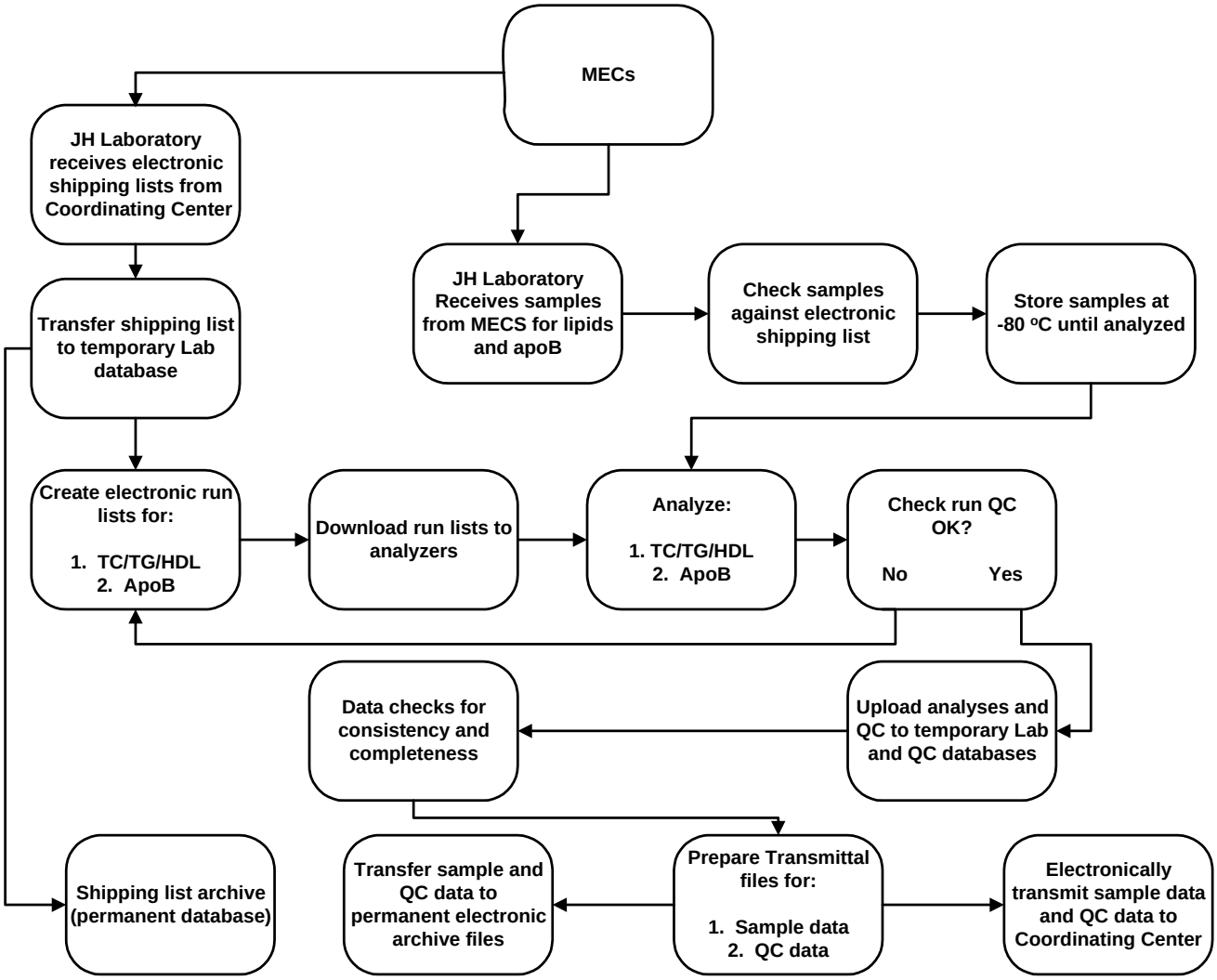
b. Blood Handling.

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., nitrile or plastic) when handling biological samples. The use of latex gloves is not allowed due to concerns for personnel having or developing latex sensitivities. Never pipet 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, 10:100, v/v) and the wipes are disposed of in a red biohazard box.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT



The NHANES Lab number is 13, and we will receive vessel(s) 21(serum).Samples will be sent to the following address via FedEx overnight shipping:

Lipoprotein Analytical Lab/JHU
Attn: Donna Virgil
600 North Wolfe Street
Blalock 1379
Baltimore, MD 21287
410-614-1030

Containers of samples will be sent from the collection locations on scheduled shipping days.

On the day the samples are shipped, our lab will receive data files in Excel format (efiles) from the database coordinating center email account. The efiles will be sent to:

Donna Virgil
dvirgil1@jhem.jhmi.edu, or dvirgil1@jhmi.edu
Ella Levy
elevy2@jhem.jhmi.edu
Cindy Wiley
cwiley2@jhmi.edu

The files will follow the file naming convention NH05_######.xls. The "NH5_" will distinguish NHANES 2005 containers from NHANES 1999 container files. The efile contains 19 pre-formatted columns.

a. Laboratory data handling.
The efile received from the database contractor email attachment is imported into the stand alone NHANES dedicated study computer. From this excel file an electronic run file is created for determining total cholesterol, triglyceride and HDL cholesterol analyses on the Hitachi 717 platform. All samples have total cholesterol and HDL cholesterol assayed. Only fasted specimens have triglycerides assayed.

The host computer for the Hitachi 717 uses a Dawning Technologies bidirectional interface to collect data from the analyzer and the program associated with it, MP Cup, will drop the data from the Hitachi 717 into a dbase file(dbf) on the host computer. The dbf is named with the current date and the data is copied to a 3.5 inch FD. The FD is then used to transfer the data to the NHANES computer. DBASE III plus is loaded on the NHANES study computer. Calculation programs to capture dbf data and drop it into the NHANES raw data report (NHRDR) have been used for the past two NHANES studies analyzed in our laboratory.

The NHRDR is visually reviewed by Donna Virgil and if corrections are necessary or a change in the default comment code of 0 is necessary, she makes them at this time.

The current DBASEIII system will output the captured data as a dbf file. This file is then imported into Excel and a comma delimited file (csv) is created. The Excel csv file is used to drop the TC, TG, and HDL-C data into the original excel shipping file received the day the samples were shipped. The specimen data transferred to the shipping file is reviewed again by both Donna Virgil and Cynthia Wiley prior to submitting results to the database coordinating center.

b. Submitting Results
Beginning with column I in Excel, the technician inserts results copied from the Excel csv file created from the NHRDR output dbf file. Not all columns will apply to every result, and those columns that do not apply should be left blank. The laboratory returns the completed results by sending the Excel attachment to the database coordinating center email account within the defined 21 day limit.

c. Result Comment Codes
Numerical comment codes are used to indicate valid results, turbidity, insufficient quantity for analysis, results less than the limit of detection, etc. The comment code is listed next to the results column for each assay value submitted

d. Updating and Deleting Results

If any results already submitted need to be updated or deleted, a change reason numerical code is used to resubmit values to the database coordinating center. No data will be changed or deleted without a change reason.
We do not need to version files each time we resend efiles to make updates or corrections. If the lab needs to correct large amounts of data that encompass many containers, we must contact the systems analyst at the database coordinating center. We can then transmit the data in one large, single file.

e. Late Results
We will receive late result email notifications from the database contractor for results that are past due. If our records do not agree with the late results email, we must contact the database contractor to define the discrepancy. If the specimen does not have a result and we must submit a comment code that most closely explains the reason for the null result (for example: vial broken), the specimen can still be marked as received.

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

a. Specimen handling

(1). Collect blood into 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 x g for 30 min at 4º 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 -20ºC, 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^{o}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.

(6) Samples are stored at -80ºC until thawed for analysis. Samples are thawed for 45 minutes on a rotating serum mixer and allowed to come to room temperature. An aliquot is first taken for TC, TG and HDLC analysis on the Hitachi 717. After Lipid analysis a second aliquot is placed in sample cups of the BNA100 for Apo B analysis. All samples verified to be on the shipment log and of sufficient volume are run. Insufficient volume is the only criteria for rejection for samples received

according to study protocol. If a shipment was delayed and the samples are received thawed, the database contractor is notified and analysis is delayed until a replacement shipment is received in the laboratory.

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

Not applicable for this procedure.

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. Instrumentation

Hitachi 717(Hitachi Global Storage Technologies, 3403 Yerba Buena Road. San Jose, California 95135

b. Other Materials

Serum mixer, transfer pipettes, sample cups, quality control normal and high serum from Solomon Park, SL2 and SL3 series.

c. Reagent Preparation

None, Rreagents are provided as working reagent solutions.

d. Standards Preparation

NA

e. Preparation of Quality Control Materials

Aliquots are thawed, mixed, and transferred to two sample cups for duplicate analysis per run.

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. A frozen serum calibration pool (Solomon Park Laboratories, Kirkland, WA) is used to calibrate total cholesterol.

a. Calibration

(1)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, presses ROUTINE, then press 3 ENTER.

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

(b) 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.

(c) 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".

(d) TESTS: Press the appropriate test keys for those tests which require a standard or standards, then press ENTER. Advance the cursor to the CALIB LOAD LIST entry field. Press 1 ENTER.

(e) 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).

(2) 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, presses ROUTINE, then press 3 ENTER.

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

(b) 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.

(c) TESTS: Activate the appropriate test or profile keys for those tests requiring a blank update, and 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".

(d) 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.

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

(f) 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)

(g) Press START to begin the calibration.

(3) Reagent/calibrator changes

(a) 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 2005-2006 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 2005-2006 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.

(b). Verification

All reagent and calibrator lots are validated with 5-10 samples run with both the old calibrator reagent run values vs. the newly calibrated channel or new reagent lot. All values must be within 5% of the older lot analysis. If the values are greater than 5% the lot is rejected for use.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. Preliminaries

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

R1 Cholesterol reagent

PIPES buffer: 75 mmol/l, pH 6.8;
Mg^{2+}: 10 mmol/l;
sodium cholate: 0.2 mmol/l;
4-aminophenazone ≥ 0.15 mmol/l;
phenol ≥ 4.2 mmol/l;
fatty alcohol polyglycol ether: 1%; cholesterol esterase (Pseudomonas spec.) ≥ 0.5 U/ml;
cholesterol oxidase (E. coli) ≥ 0.15 U/ml; peroxidase (horseradish) ≥ 0.25 U/ml;
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^{o}C, or 7 days at room temperature. Protect the cholesterol reagent from light. 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.

b. Sample preparation
Samples are thawed and mixed. Sample is then transferred to sample cup and placed in the sample wheel for analysis.

c. Instrument setup
Instrument Settings:

(1) Cholesterol	Temperature: 37°C
Test	[CHOL]
Assay Code	[1POINT]:[24]-[0]
Sample Volume	[3] [2]
R1 Volume	[250][100][NO]
R2 Volume	[0][20][NO]
Wavelength	[700][505]
Calib. method	[LINEAR][0][0]
Std. (1) Conc.-Pos.	[0]-[1]
Std. (2) Conc.-Pos.	[*]-[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]-[239]
TECH Limit	[0]-[800]
Instrument Factor	[1.00]

[*] Enter the lot specific calibrator value

d. Operation of Assay Procedure
Specimen About 300 µl of serum, fresh frozen at -70°C, from fasting subjects (minimum 9 hours) is required. The Hitachi analyzer is calibrated at the beginning of the week and as necessary thereafter. A one point calibration procedure is used for Direct HDL-cholesterol. Frozen serum calibration pools (Solomon Park Laboratories, Kirkland, WA) are used to calibrate total cholesterol. The direct method HDL-cholesterol assays uses Lipids Cfas(calibrator for automated systems) available from Roche Diagnostics. Triglycerides are calibrated using calibration sera (Cfas) obtained from the Roche Diagnostics

(1). Daily Check
The following procedures are performed at the beginning of each work day before the first analytical run.
(a). Check water supply.
(b). Check 2% Hitergent supply**.

(c). Check Cell Clean 90 supply**.
(d) Prepare reagents, controls and calibrators as needed.
(e). Exchange incubation bath water: Press MAINTENANCE, then press 1 E ENTER.
(f). Perform Photometer Check.
(g). Air purge: "Start Conditions" display.
(h). Wipe sample and reagent probes.
(i). Probe adjust.

** Refer to Section 6. Reagents, above

(2) 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.

(a) CALIBRATION TYPE: Press 1 ENTER to specify "Start-Up" Calibration.
(b) 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.
(c) 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".
(d) TESTS: Press the appropriate test keys for those tests which require a standard or standards, then press ENTER. Advance the cursor to the CALIB LOAD LIST entry field. Press 1 ENTER.
(f) 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).

(3).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.
(a) CALIBRATION TYPE: Press 1 ENTER to specify "Start-Up" Calibration.
(b) 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.
(c) 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".
(d) TESTS: Deselect all previously selected tests so that no tests are selected for "STD 2-6", then press ENTER.

(e) Press ROUTINE, then press 4 ENTER and the Start Conditions screen will appear on the display.
(f) 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)
(g) Press START to begin the calibration.

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e. Recording of Data
Data is transferred to a 3.5 floppy disk as a dbf file and imported into a dedicated stand alone PC which imports the data into the raw data report. At this point a visual review of the data is done prior to exporting the data to another dbf format file which is then opened in excel. The excel file will be edited to include rundate, runnumber and technician number and a csv file is created. From the comma delimited file the data is copied and pasted into the original excel spreadsheet that is received electronically prior to sample shipment. Once all analyses are compiled into the shipment file it is transmitted to the database coordinating center via email attachment. Each datafile is acknowledged with a processing status reply email letting the lab know if the data imported into the dbase correctly. If notification is made that the file needs editing then edits are made and the file resent and renamed with a higher version number.

f. Calculations
The Hitachi 717 microcomputer uses absorbance measurements to calculate cholesterol concentrations as follows:
$C_X = [K(A_X - A_b) + C_b] \times IF$
Where:
C_X = Concentration of Sample.
K = Concentration factor (determined during calibration).
A_X = Mean of absorbances of Sample + R1 read during cycles indicated in Assay Code field for the respective test.
A_b = Mean of absorbances of Blank +R1 read during cycles indicated in the Assay Code field for the respective test.
C_b = Concentration of Blank (STD).
IF = Instrument Factor (dilution correction
IF = 1.00 for cholesterol.

9. REPORTABLE RANGE OF RESULTS

LINEARITY: 3 - 800 mg/dL

When the result exceeds 800 mg/dL the specimen is to be diluted with 0.9% NaCl and reassayed.

Report patient results less than 3 mg/dL as 3 mg/dL with a comment code of 37 and 3 as the LOD.

10. 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.

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 In each case, one pool has normal, and the other elevated concentrations of the respective analytes. An aliquot from each pool is analyzed two 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.

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 2005-2006, n = 2.
The overall mean for the pool, $\underline{X}$, is calculated by summing the individual run means and dividing by the number of runs, N:
$\underline{X}$ = 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:
$\underline{R}$ = 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 = $\underline{X} + 3S_X$
Lower control limit = $\underline{X} - 3S_X$

Control limits are rounded to the nearest whole number.
The warning (95%) limits for the X chart are calculated as follows:
Upper warning limit = $\underline{X} + 2S_X$
Lower warning limit = $\underline{X} - 2S_X$

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 = $\underline{R} + 3\ S_r$
Range warning limit = $\underline{R} + 2\ S_r$
Where S_r is the standard deviation of $\underline{R}$.
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 ($\underline{X} \pm 3S_X$) 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 $\underline{X}$, S_X, 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 is calculated as the algebraic difference between the $\underline{X}$ 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 $\underline{X}$ and R are arranged one above the other on the same sheet of graph paper. Draw the $\underline{X}$ 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 $\underline{R}$ line and R limits on the $\underline{R}$ 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.

Table 1. Precision and Accuracy of QC pools

Pool	Mean	95% limits	99% limits	95% limits (range)	99% limits (range)	Cumu-lative Mean	N
SL2 I348	177	2SD = 6.02	3SD = 9.03	170.98-183.02	167.97-186.03	177.0	121
SL3 I180	245	2 SD = 6.0	3SD = 9.0	239-251	236-254	244.5	999

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

a. 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.

b. 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 717 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.

12. LIMITATIONS OF CHOLESTEROL METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The following information applies to cholesterol

a. Icterus: No significant interference up to an I index of 25 (approximate conjugated bilirubin concentration: 25 mg/dL or 428 μmol/L) and an I index of 10 (approximate unconjugated bilirubin concentration: 10 mg/dL or 171 μmol/L).

b. Hemolysis: No significant interference up to an H index of 700 (approximate hemoglobin concentration: 700 mg/dL or 435 μmol/L).

c. Lipemia (Intralipid): No significant interference up to an L index of 1250. There is poor correlation between the L index (corresponds to turbidity) and triglycerides concentration.

d. In very rare cases gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

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

e. 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 a-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.

13. REFERENCE RANGES (NORMAL VALUES)

The ranges we use for total cholesterol are as shown below.

	Age ≤ 12 y	Age >12 y
cholesterol	100-300 mg/dL	140-400 mg/dL

14. CRITICAL CALL RESULTS ("PANIC VALUES")

For Total cholesterol a value of 400 or more, though not considered life threatening, is reported to the medical officer.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens remain frozen at -80ºC until thawed and mixed just prior to analysis. Samples are stored at 4ºC until all assays are performed within control limits. All specimens are tested within 24 hours of thaw.

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

Samples are held at -80ºC in the freezer in 1379. If a problem occurs and this freezer begins to warm. Samples are transferred to the research freezers located in 1358. A service call is placed to repair the freezer in 1379. A loaner freezer is requested for each service repair that removes the freezer from 1379 for any period greater than 1 day.

No alternate test site has been identified. As far as downtime for equipment repairs, the 21 day turnaround time as established in the contract, has always been sufficient enough to allow the repair to occur prior to the deadline for sample analysis. If the repair could not be accomplished in the time frame allowed we will discuss the three options available to us with the project officer. One option is to wait until the repair is made if the proposed repair date is agreeable to the project officer. The second option is to perform the analyses in the Clinical Chemistry Laboratory of the Johns Hopkins Hospital. While the chemistries are identical, they are performed in the Clinical Chemistry laboratory on a larger platform, namely the Hitachi Modular. While this laboratory is CLIA certified it is not a participant in the CDC LSP program. Split sample comparisons are run between the two laboratories on patient samples so relative bias is known. The third option would be to use the NWRL since it is a CDC referenced laboratory. If necessary the JHU and NWRL laboratories can establish the bias between the two laboratories.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

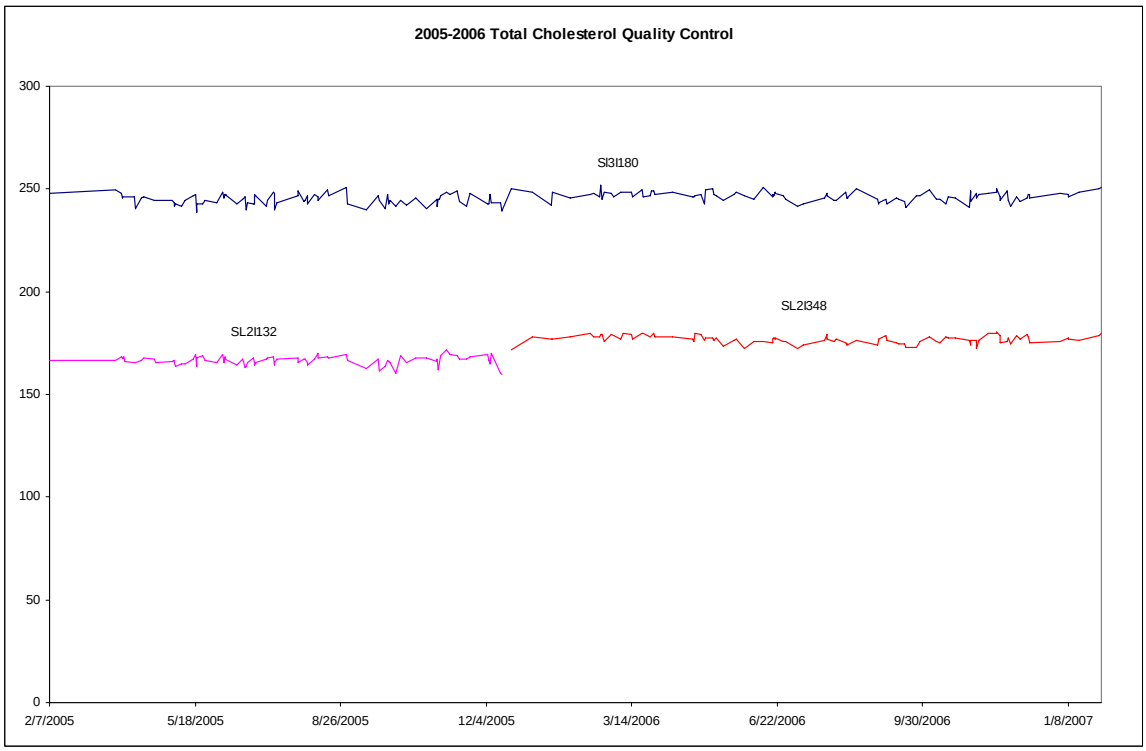
Critical action values are called into the Medical Officer Kathryn Porter for Cholesterol values greater than 400.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

19. SUMMARY STATISTICS AND QC GRAPHS

Summary Statistics for Total Cholesterol by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SL2I132	87	2/7/2005	12/15/2005	166.4	2.2	1.3
Sl3I180	187	2/7/2005	1/31/2007	245.7	2.6	1.1
SL2I348	100	12/21/2005	1/31/2007	176.8	1.9	1.1



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