Laboratory Procedure Manual

Analyte: HDL- Cholesterol

Matrix: **Serum**

Method: **Hitachi 912 Analyzer**

as performed by: *Lipid Laboratory Johns Hopkins*

University School of Medicine

Lipoprotein Analytical Laboratory

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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 (and SI units)
HDL_D	LBXHDD	HDL-Cholesterol (mg/dL)
	LBDHDDSI	HDL-Cholesterol (mmol)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

    HDL-cholesterol is measured directly in serum without the need to remove the apoB containing lipoproteins.

    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).

    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 dextran sulfate 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.   Enzymatic colorimetric test.: 1st incubation step (sample + R1)

    It is likely that in the presence of slightly alkaline buffer and magnesium sulfate and dextran sulfate selectively form water-soluble complexes with LDL, VLDL, and chylomicrons, which are resistant to PEG-modified enzymes.

    2nd incubation step (sample + R1 + R2): The cholesterol concentration of HDL cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (approx. 40%).
    Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase Δ^4 cholestenone and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-amino-antipyrine and HSDA to form a purple-blue dye. The color intensity of this dye is proportional to the cholesterol concentration and can be measured photometrically.

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 of aerosols, do not leave samples open to the atmosphere longer than necessary.
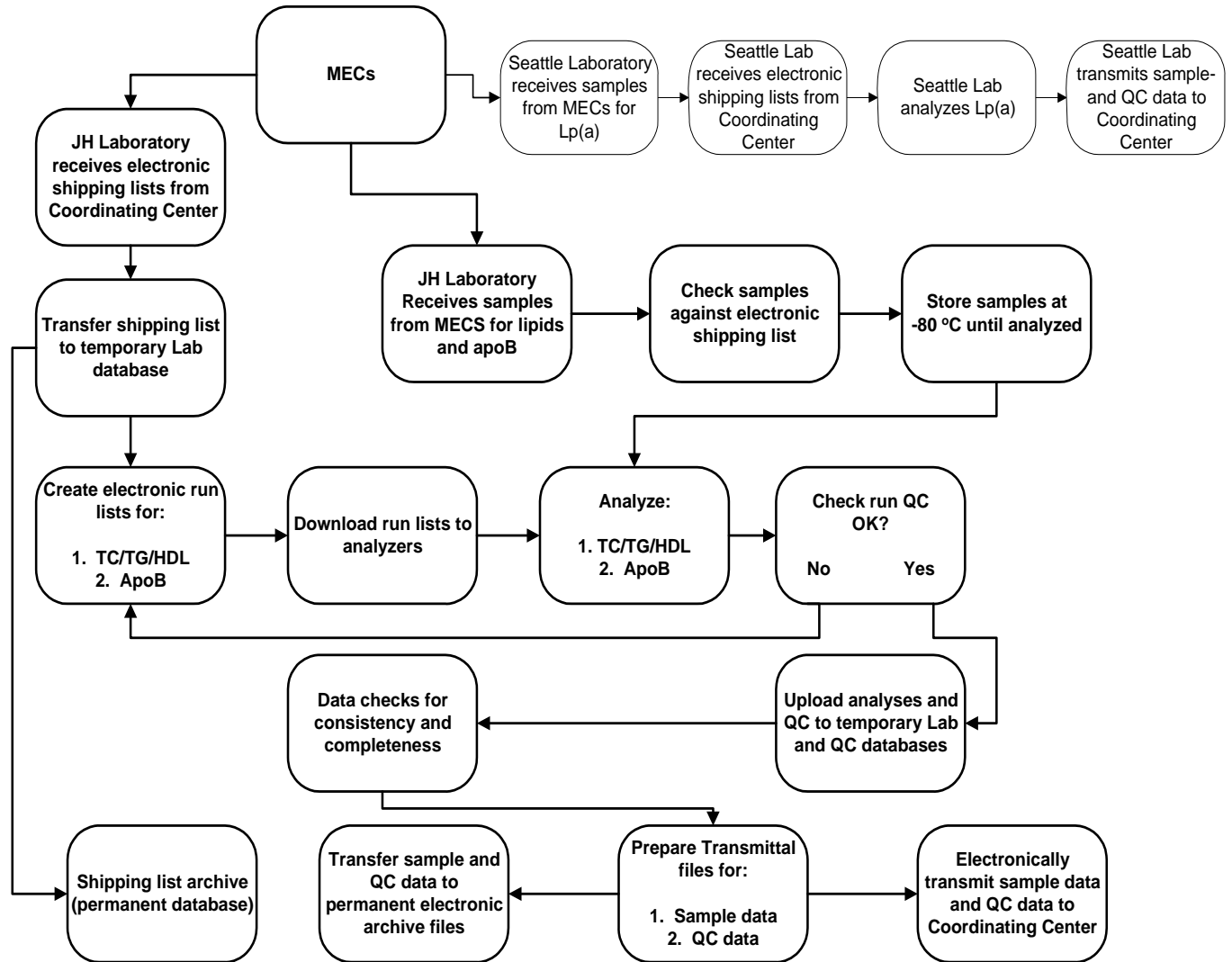
It is about 30 times easier to become infected with hepatitis than with HIV through sample mishandling, and it has been recommended that the usual precautions for handling blood specimens to prevent hepatitis infection serve as a guide to prevent AIDS infection as well. Handle all specimens as if you 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

HDL-cholesterol in Serum using Hitachi 912
NHANES 2005-2006

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                                    ┌─────────────┐     Seattle Laboratory  Seattle Lab         Seattle Lab
                                    │    MECs     │     receives samples    receives electronic  Seattle Lab       transmits sample-
                                    │             │     from MECs for       shipping lists from  analyzes Lp(a)    and QC data to
                                    └─────────────┘     Lp(a)               Coordinating                          Coordinating
                                                                           Center                                Center

┌──────────────┐
│ JH Laboratory│
│   receives   │
│  electronic  │
│shipping lists│
│     from     │
│ Coordinating │
│    Center    │              ┌──────────────┐    ┌──────────────┐    ┌──────────────┐
└──────────────┘              │ JH Laboratory│    │Check samples │    │Store samples │
                              │Receives      │───▶│   against    │───▶│     at       │
┌──────────────┐              │samples from  │    │  electronic  │    │-80 ºC until  │
│Transfer      │              │MECS for      │    │shipping list │    │  analyzed    │
│shipping list │              │lipids and    │    └──────────────┘    └──────────────┘
│to temporary  │              │apoB          │
│Lab database  │              └──────────────┘
└──────────────┘

┌──────────────┐    ┌──────────────┐    ┌──────────────┐    ┌──────────────┐
│Create        │    │Download run  │    │  Analyze:    │    │Check run QC  │
│electronic run│───▶│lists to      │───▶│              │───▶│     OK?      │
│lists for:    │    │analyzers     │    │1. TC/TG/HDL  │    │              │
│1. TC/TG/HDL  │    └──────────────┘    │2.  ApoB      │    │No      Yes   │
│2.  ApoB      │                        └──────────────┘    └──────────────┘

                    ┌──────────────┐                        ┌──────────────┐
                    │Data checks   │                        │Upload        │
                    │for           │◀───────────────────────│analyses and  │
                    │consistency   │                        │QC to         │
                    │and           │                        │temporary Lab │
                    │completeness  │                        │and QC        │
                    └──────────────┘                        │databases     │
                                                            └──────────────┘

┌──────────────┐    ┌──────────────┐    ┌──────────────┐    ┌──────────────┐
│Shipping list │    │Transfer      │    │Prepare       │    │Electronically│
│archive       │    │sample and    │    │Transmittal   │    │transmit      │
│(permanent    │    │QC data to    │◀───│files for:    │───▶│sample data   │
│database)     │    │permanent     │    │              │    │and QC data to│
└──────────────┘    │electronic    │    │1. Sample data│    │Coordinating  │
                    │archive files │    │2. QC data    │    │Center        │
                    └──────────────┘    └──────────────┘    └──────────────┘
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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.

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 912 platform. All samples have total cholesterol and HDL cholesterol assayed. Only fasted specimens have triglycerides assayed.

The new host computer for the Hitachi 912 uses the Hitachi 912 computer interface cabled to it to collect data from the analyzer. The operating program purchased to act as the host computer interface, Cache Intersystems, will drop the data from the Hitachi 912 into an excel file on the host computer. The excel file will be linked to the efile received and the data copied to the appropriate specimen field.   The efile 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 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.

Submitting Results
Beginning with column I in Excel, the technician inserts results copied from the Excel csv file created by the host computer. 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.

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

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.

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 (for example: vial broken) that most closely explains the reason for the null result, 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 4ºC. 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 -80ºC. 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 912. 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 912

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, reagent provided as working reagent solution.

D.   Preparation of Quality Control Materials

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

7.   CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A.   Calibration Curve

Standardization: The homogeneous HDL-cholesterol method has been calibrated against the designated CDC reference method (designated comparison method). The standardization meets the requirements of the "HDL Cholesterol Method Evaluation Protocol for Manufacturers" of the US National Reference System for Cholesterol, CRMLN (Cholesterol Reference Method Laboratory Network), November 1994.
S1: 0.9% NaCl
S2: C.f.a.s. (Calibrator for automated systems), Lipids, Cat. No. 2172623
Calibration frequency – Two-point calibration is recommended.
- after reagent lot change
- as required following quality control procedures

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

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.

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.

B. Sample preparation

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.

C. Instrument setup

Direct HDL-cholesterol	Temperature: 37°C
Test	[HDLC2]
Analyzer cycle time	10 sec
Application code	035
Assay Code	[2POINT]:[24]-[50]
Sample volume	[3] [3]
R1 Volume	[250][0][NO]
R3 Volume	[83][0][NO]
Wavelength	[700][600]
Calib. Method	[LINEAR][0][0]
Std. (1) Conc.-Pos.	[0]-[1]
Std. (2) Conc.-Pos.	[*]-[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	[90]
Sensitivity Limit	[600]
ABS. Limit (INC/DEC)	[0][INC.]
Prozone Limit	[32000][UPPER]
Expected Value	[60]-[120]
TECH Limit	[0]-[120]
Instrument Factor	[1.00]

[*] Enter the lot specific calibrator value

    D. Operation of Assay Procedure

Approximately 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

    Daily Check/ Power Up
    a. Check levels of detergents
    b. Ensure external water supply is on
    c. Empty waste container.
    d. Check printer paper supply
    e. Power on or wake up Hitachi 912
    f. Log on to the system
    g. Check reagents screen for inventory

2. Start Up procedures
    a. Clean and adjust sample and reagent probes.
    b. Clean stir paddles.
    c. Perform Incubation Bath exchange.
    d. Perform Photometer Check
    e. Place 500 µL of System Cleaning Solution in W1 and 500 µL 4N NaOH in W2 on the sample tray.
    f. Schedule calibration if necessary
    g. Press <Print> to print an appropriate calibrator load list
    h. Load calibrators and controls.
    i. Verify start conditions
    j. Press Start.

3. 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 <u>not</u> 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. 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.

    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

  4. 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, 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.
   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.

 5. 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.

 6. Calculations

The Hitachi 912 microcomputer uses absorbance measurements to calculate HDL – 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 direct method HDL-cholesterol.

9. REPORTABLE RANGE OF RESULTS

    LINEARITY: 3 - 120 mg/dL

    Report patient results less than 3 mg/dL as <3 mg/dL.

    REFERENCE RANGE: 35-96 mg/dL

10. QUALITY CONTROL (QC) PROCEDURES

    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 28 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 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:
$S_X = \quad (x - x)^2/(N - 1)$

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, triglyceride or HDL-cholesterol measurement 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.

H..Actions taken when analyses are not acceptable.

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.

I. Standardization and External Surveillance

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 ($\pm$ 1 week to account for unforeseen delays). The data will be transmitted to CDC and evaluated upon completion of all four runs.

The laboratory also participates in the College of American Pathologists Chemistry Survey for Direct HDLC. This proficiency survey involves 5 pools for the lipid analytes
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Table 2. Precision and Accuracy of HDL Control Pools

Pool	Mean	95% limits	99% limits	95% limits (range)	99% limits (range)	Runs
SL4 I267	28.0	2SD = 2.0	3SD = 3.0	26-30	25-31	430
SL4 I236	49.9	2 SD = 2.26	3SD = 3.39	47.6-52.2	46.51-53.3	454

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 as "in control" , 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 912 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 limits are created, temporary or permanent.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Perform a cell wash daily with SMS/Acid Wash to prevent magnesium hydroxide from being
deposited in the cell wash tubing line.

Criterion: Recovery within ± 10% of initial value.
Icterus: No significant interference up to an I index of 30 for conjugated bilirubin and 70 for
unconjugated bilirubin (approximate conjugated bilirubin concentration: 30 mg/dl or 513
µmol/l; approximate conjugated bilirubin concentration: 70 mg/dl or 1197 µmol/l).

These claims are based on the Glick model. Please refer to further comments below
(abnormal liver function).

Hemolysis: No significant interference up to an H index of 1200 (approximate hemoglobin
concentration: 1200 mg/dl or 745 µmol/l).

Lipemia (Intralipid): No significant interference up to an L index of 1000. No significant
interference from native triglycerides up to 1200 mg/dl. There is poor correlation between
turbidity and triglycerides concentration.

The claim for lipemia interference is based on the Glick model, which uses Intralipid as an
artificial substrate. To date, there is no model available which can mimic interference by
triglycerides, as triglyceride levels in patient specimens behave unpredictably, depending on
the nature of the esterified fatty acids in the samples. Patient specimens with elevated
triglyceride levels are very often lipemic. Therefore customers cannot verify interference by
triglycerides in patient specimens.

Elevated concentrations of free fatty acids and denatured lipoproteins may cause falsely
elevated HDL-cholesterol results.

Ascorbic acid up to 50 mg/dl does not interfere.

In rare cases, elevated immunoglobulin concentrations can lead to falsely increased HDL-cholesterol results. Abnormal liver function affects lipid metabolism; consequently, HDL and LDL results are of limited diagnostic value. In some patients with abnormal liver function, the HDL-C plus result is significantly negatively biased versus the DCM (designated comparison method)result. Of 20 commonly used drugs tested in vitro, none interfered with the assay.

13. REFERENCE RANGES (NORMAL VALUES)

	Age ≤ 12 y	Age >12 y
HDL-Cholesterol	20-85 mg/dL	20-85 mg/dL

14. CRITICAL CALL RESULTS ("PANIC VALUES")- .

There are no critical call results for this analyte.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens remain frozen at -80º C until thawed and mixed just prior to analysis. Samples are stored at 4º C 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. T he 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 is 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.

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

There are no critical call results for this analyte.

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

The shipping list that is emailed to the laboratory is used to create runs for the Hitachi Chemistry Analyzer. Shipments are checked against the email shipping lists upon arrival in the laboratory. Problems with vials such as condition, QNS etc are noted upon physical inspection. The number comment codes provided to the laboratory by The database contractor and NCHS for reporting data are used to indicate individual analyte comments. For example, if the individual vial was empty upon inspection then the empty vial code of 18 is entered in the comment field for each analysis requested for that individual specimen ID. The report form for NHANES 2005 is an Excel spreadsheet sent originally as the shipping list email attachment with the data entry columns blank. Data is transferred from the instruments to the spreadsheet and visually checked for transcription errors by the Lab and Study Coordinators prior to email transfer to database coordinating center. The laboratory has 21 days from the receipt of samples in the laboratory to report the specimen data to the database coordinating center. Should the laboratory exceed the 21 day contractual limit, the database contractor notifies the Study Coordinator by email of the individual specimens and the test data owed for each specimen.

19. SUMMARY STATISTICS and QC GRAPHS

Summary Statistics for HDL Cholesterol by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SLI267	91	2/7/2005	1/31/2006	28.0	0.6	2.0
SL4I236	186	2/7/2005	1/31/2007	50.4	0.9	1.9
SLI438	95	2/14/2006	1/31/2007	26.6	0.7	2.6

2005-2006 HDL Cholesterol Quality Control
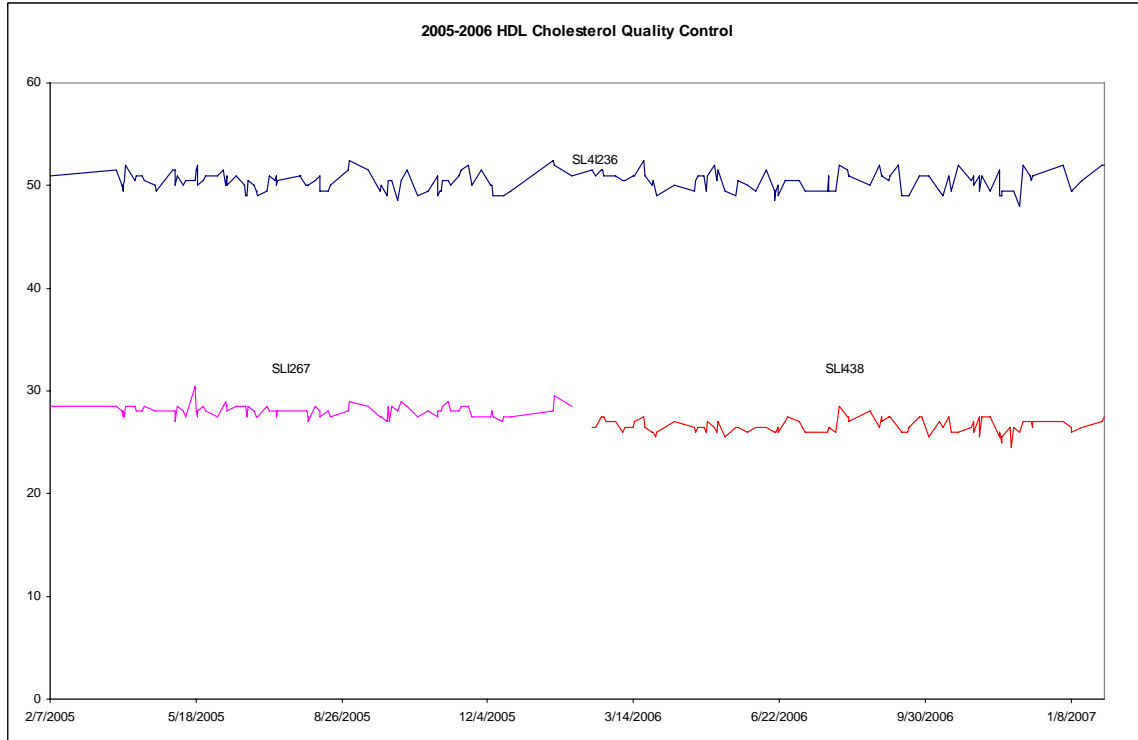
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