



Add Health

The National Longitudinal Study of Adolescent to Adult Health

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Lipids User Guide

This document summarizes the rationale, equipment, protocol, assay, internal quality control, data cleaning, external quality control, and procedures for the measurement and classification of lipid concentrations at the Wave VI home exam. Whenever possible, data collection and methods in Wave VI mirrored those of Wave V to ensure comparability of data between waves, although **important inter-Wave differences between Waves IV-VI exist and are grey-highlighted herein**. This document is one in a set of Wave V user guides. User guides are also available to describe protocols for the following biological measures at Wave V:

- Anthropometry
- Baroreflex Sensitivity & Hemodynamic Recovery
- Biomarker Weights
- Cardiovascular Measures
- Glucose Homeostasis
- Hepatic Injury
- Home Exam – Medication Use
- Home Exam Questionnaire and QC Metrics
- Infection
- Inflammation and Immune Function
- Neurodegeneration
- Renal Function

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1. Introduction

Wave IV lipid concentrations were measured using dried blood spots collected using capillary finger prick¹. In contrast, Wave V-VI lipid concentrations were based on venous blood collected via phlebotomy. Assay methods at Wave V² also differed from those at Wave VI, as highlighted here and below. The blood was collected by field examiners (FEs) certified in phlebotomy, chilled at 4°C during the remainder of the home exam, centrifuged immediately afterward, aliquoted into transport tubes, and then sent overnight to a laboratory for assay.

Assayed Lipid Concentrations

- Total cholesterol (TC, mg/dl)
- High-density lipoprotein cholesterol (HDL-C, mg/dl)
- Triglycerides (TG, mg/dl)
- Low-density lipoprotein cholesterol (LDL-C, mg/dl), if TG > 400 mg/dl

Moreover, the restricted use Add Health Wave VI data include nineteen constructed measures designed to facilitate analysis and interpretation of lipid concentrations:

Constructed Lipid Concentrations and Ratio

- Low-density lipoprotein cholesterol³ (LDL-C, mg/dl), if TG ≤ 400 mg/dl
- Non-high-density lipoprotein cholesterol⁴ (non-HDL-C, mg/dl)
- Total to high-density lipoprotein cholesterol ratio⁵ (TC:HDL-C)

Lipid Classifications

- According to decile rank
- According to NCEP/ATP III guidelines⁶
- According to AHA/ACC guidelines⁷

Other

- LDL-C flag indicating the method used to generate the result
- Fasting time (hr)
- Fasting status flag indicating a status of nine hours or more
- Antihyperlipidemic medication use
- Hyperlipidemia joint classifications

2. General Overview of Data Collection

All Wave VI venous blood samples were collected during home exams performed by Exam One, a subsidiary of Quest Diagnostics®. All FEs were trained and certified using a custom program specific to the Add Health protocol. FEs used a 7" Samsung Galaxy Tab A7 Lite tablet to record and transmit data. An Add Health data collection application (Open Data Kit or ODK) installed on the tablet guided the FEs through the home exam protocol. In addition, FEs received a series of job aids, both on paper and on the tablet, to serve as quick reference guides when completing the protocol. Each tablet also contained an in-depth Add Health training manual that could be accessed at any time.

FEs conducted home exams among previously consented participants. All FEs were phlebotomy-certified and had at least two years of experience collecting venous blood. Before home exams, FEs were sent a Visit Supply Kit that included a box for shipping blood to the lab and a Blood Collection Kit containing most required materials for the blood collection. FEs supplied additional materials, as needed (see section 3.2). Protocols for blood collection were dictated to FEs by the handheld 7" Samsung tablet used during all home exams. The tablet gave step-by-step directions for the blood collection and required FEs to enter information about the blood draw for each participant. All participants had the option to decline part or all of the blood draw, although declining did not affect their ability to participate in the rest of the home exam. Overall, 90.8% of the participants agreed to and completed the blood draw. Of the remainder, 6.4% refused, 2.1% agreed but the blood draw was unsuccessful, and < 1% had exams terminated before the blood draw (see the blood draw status variable H6BLOOD in the *bdemo6* data set and codebook).

Blood collection was the last step in the home exam. Afterwards, all collection tubes were inverted 8-10 times to distribute the blood and contents of the tubes and then chilled at 4° C (on ice or frozen cold packs) for up to two hours. Subsequent processing involved centrifuging specific tubes then aliquoting serum and plasma into color-coded transport tubes pre-labelled with unique barcode identifiers linking the blood to a particular participant. Then the transport tubes were packaged in a Styrofoam Box with frozen cold packs and shipped overnight via FedEx to the Laboratory for Clinical Biochemistry Research (LCBR) at the University of Vermont. Overnight shipment enabled receipt by LCBR before 10:30 a.m. the next morning. Upon receipt, LCBR documented the arrival of the transport tubes, evaluated their condition, processed them, and either assayed the specimens or aliquoted and archived them in -80°C freezers.

3. Blood Collection

3.1 Rationale

Venous blood was collected to provide Add Health with the biological specimens necessary to assay and interpret pre-specified biomarkers of metabolic, hepatic, renal, amyloid-tau-neurodegenerative (ATN), inflammatory, immune, and infectious conditions, including the lipids described herein. It also was collected to establish an archive of serum, plasma, whole blood, RNA, and packed cells capable of supporting future assays and ancillary studies.

3.2 Equipment

Before exams, FEs were shipped a Visit Supply Kit (**Figure 1**) including (1) a cardboard Shipping Box with an inner Styrofoam Box and two cold packs for shipping collected samples to LCBR, (2) a large Tyvek envelope in which to ship the Shipping Box, and (3) a Blood Collection Kit for collecting blood. The Blood Collection Kit contained:

- Biohazard-labelled Ziploc bag
- Latex-free gloves
- 2"x2" gauze
- Latex-free, Band-Aid type adhesive dressings
- Latex-free, strap tourniquet
- Alcohol prep pads, disposable pipets
- Single-use vacutainer (blood collection) tube holder
- 21-gauge Eclipse straight needle
- 21-gauge butterfly needle
- (3) disposable 3 ml graduated transfer pipets
- (2) 8.5 ml serum separation transport (SST) vacutainer tubes
- (1) 3 ml potassium ethylenediaminetetraacetic acid (EDTA)-containing vacutainer tube
- (1) 10 ml EDTA-containing vacutainer tube
- (1) 10 ml PAXgene vacutainer tube (containing 7.5 ml of preservative)
- (4) 10 ml transport tubes with color coded caps
- Extra barcode labels

BD Biosciences (San Jose, CA) supplied all vacutainer tubes, and transport tubes were supplied by Simport Scientific (Quebec, Canada).



Figure 1. Visit Supply and Blood Collection Kits

FEs were responsible for providing ancillary materials for each home exam, including but not limited to a chux-type absorbent under pad, a sharps container, and a cooler with cold packs for keeping samples cold before packaging and shipping them to LCBR.

3.3 General Protocol

3.3.1 Blood Collection

The blood draw was performed as the final stage of the home exam following collection of anthropometric, cardiovascular, and medication information. After confirming participants were comfortable giving blood, participants were asked to either sit or recline at their discretion. They also were asked if they had problems in the past with blood collection such as fainting, bleeding, or hard-to-find veins. FEs were instructed to ensure the blood collection area was private, uncluttered, and fully prepared before beginning the blood draw. Preparation involved placing the chux pad, organizing the vacutainer tubes/supplies, preparing the cooler to accept the blood samples, and scanning the barcode located on the outside of the Blood Collection Kit and on all vacutainer tubes. Scanning it automatically captured a unique, eight-digit code, thereby linking the participant to the transport tubes / labels within it, the corresponding ODK questionnaire data, and ultimately to LCBR results.

Following standard phlebotomy protocols, FEs asked participants to identify an arm for collecting blood, applied the tourniquet to that arm, and identified a vein in the antecubital fossa for venipuncture. If no vein appeared suitable, FEs asked to try the opposite arm. Unless participants had objections, venipuncture was performed on the best potential vein and whole blood was collected, as summarized below:

- Put on nitrile gloves.
- Have the participant extend his/her arm on the protective pad, palm up and straight at the elbow.

- Inspect the arm. Do not draw blood from an arm that has a rash, open sore, is swollen or shows signs of a recent venipuncture or hematoma. Do not draw blood from an arm that contains an arterial access such as a fistula or shunt.
- Apply the tourniquet several inches above the elbow and palpate for a suitable vein.
- Select a vein that is palpable and well-fixed to surrounding tissue.
- Open the needle assembly unit and attach it to the vacutainer holder.
- Ask the participant to make a tight fist. Cleanse the area with an alcohol wipe using a circular motion and allow the area to air dry.
- Remove the cover from the needle.
- The vein should be fixed or held taut during the puncture. Push the needle firmly and deliberately into the vein. When firmly in the vein, blood appears in the tubing of the needle assembly past the end of the needle.
- Attach the needle holder and quickly push the first vacutainer tube (ordered in Figure 2, below) onto the needle in the holder, puncturing the center of the stopper.
- Release the tourniquet after the flow is established or if the participant becomes uncomfortable. The participant may open his/her fist once blood flow is established.
- When the first vacutainer tube is filled to capacity, remove it from the holder and place the next vacutainer tube in the holder.
- Gently invert each vacutainer tube 8-10 times immediately upon removing each one and while filling the next one. Repeat until all the desired vacutainer tubes are filled.
- Place all filled vacutainer tubes directly into a cooler with ice or ice packs.
- When the last vacutainer tube is filled, remove the tourniquet, carefully withdraw the needle, and cover the venipuncture site with a sterile gauze pad.
- Never apply pressure to the gauze until the needle is clear of the puncture site and away from the arm.
- Have the participant hold the gauze pad with mild pressure and sit quietly for a few minutes.
- Slide the needle safety guard forward to prevent an accidental needle stick. Discard the entire used needle assembly in a sharps container.
- Check the venipuncture site. If it is adequately clotted, remove the gauze and apply a bandage. If after a few minutes, bleeding continues keep direct pressure on the site for 5 minutes.
- Encourage the participant to sit quietly for a few minutes. Due to a fasting blood draw encourage the participant to eat a snack if needed.

When the first attempt at blood collection was unsuccessful, FEs were allowed to ask to draw blood from the opposite arm. However, no more than two blood collection attempts were permitted. Moreover, only the antecubital fossa was acceptable for blood draw. FEs were not allowed to collect blood from any other sites, such as the back of the hand.

5 tubes of blood were collected per participant. Collection order, tube type, and processing information are listed below (**Figure 2**).

Order	Tube Type	Centrifuged	Resultant supernatant	Resultant precipitate	Use
1	8.5 ml SST	Yes	Serum	Discarded	Assay: glucose, total cholesterol, high- & low-density lipoprotein-cholesterol, triglycerides, AST, ALT, creatinine, hsCRP, IL-1 β , IL-6, IL-8, IL-10, TNF α , CMV, HSV, SARS CoV-2 (RBD; spike; nucleocapsid) IgG
2	10 ml EDTA	Yes	Plasma	Packed cells	Assay: Neurofilament light, Tau, GFAP. Archival: packed cells for future use
3	3 ml EDTA	No	N/A	N/A	Assay: hemoglobin A1c Archival: for future use.
4	8.5 ml SST	Yes	Serum	Discarded	Archival: for future use
5	10 ml PAXgene	No	N/A	N/A	Archival: for future use

Figure 2. Tubes of Blood Collected

3.3.2 Blood Processing

The venous blood draw concluded the home exam. After cleaning up all supplies and equipment, FEs left the exam sites and were allowed a maximum of two hours before processing the blood which was chilled at 4° C (on ice or frozen cold packs) in the interim.

All FEs centrifuged the 8.5 ml SST and 10 ml EDTA vacutainer tubes. The 3 ml EDTA vacutainer tube used for the HbA1c assay and the PAX gene tube were *not* centrifuged. FEs centrifuged tubes for ≥ 10 min at ≥ 1300 g, depending on the capabilities of their centrifuge. After centrifugation, FEs used the graduated transfer pipettes included in the Blood Collection Kit to aliquot serum from the SST tubes and plasma/packed cells from the 10 ml EDTA tubes into 10 ml, round bottom, skirted transport tubes (BD Biosciences, NJ). FEs aliquoted as much supernatant as possible into the transport tubes but avoided disturbing the precipitate layer. A red cap identified transport tubes containing serum from the SST vacutainer tubes and a blue cap identified transport tubes containing plasma from the 10 ml EDTA vacutainer tube. Transport tubes were chilled at 4° C (on ice or frozen cold packs) until packaged for shipment to LCBR. **Figure 3** demonstrates the complete blood processing protocol.

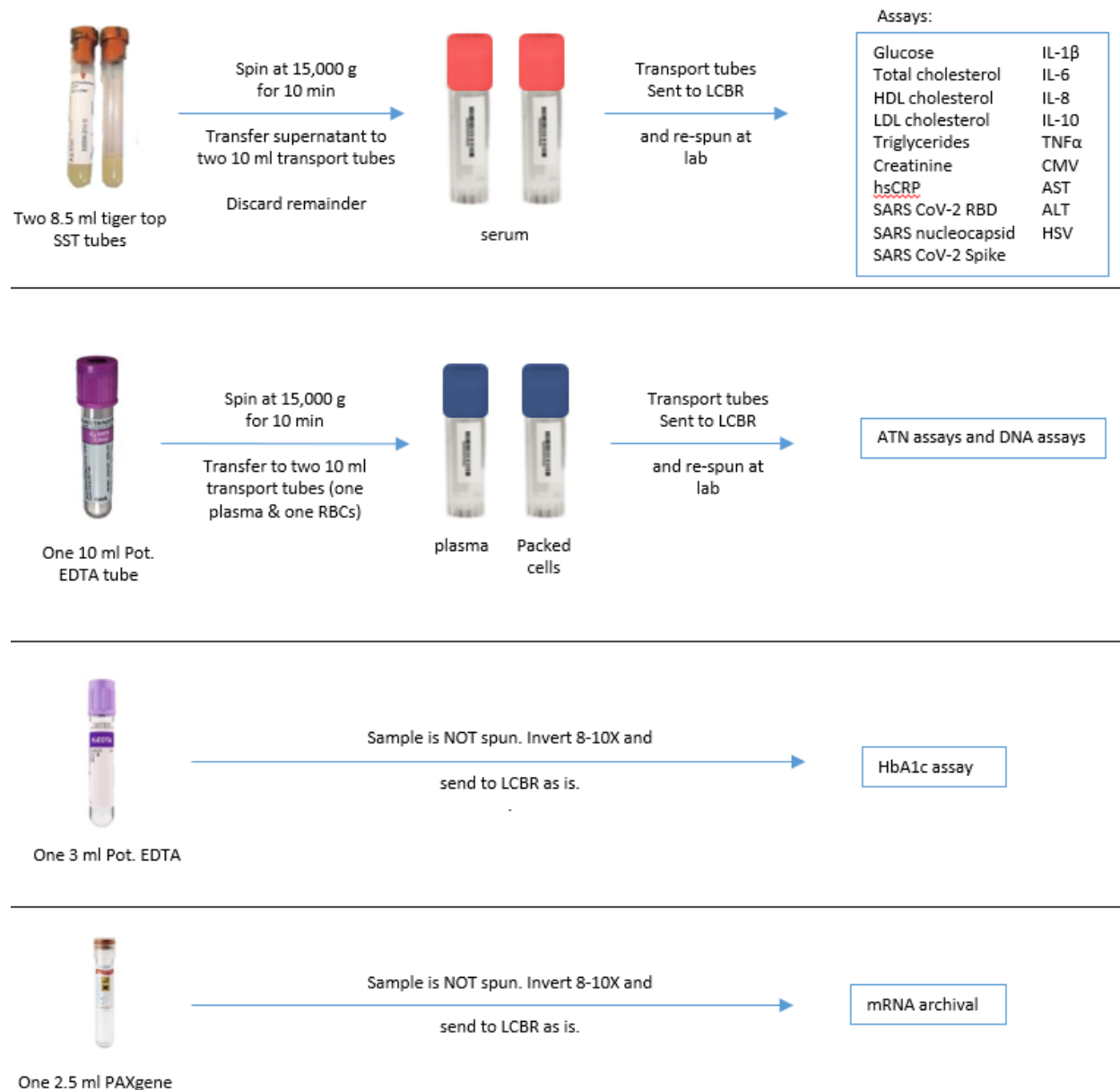


Figure 3. Blood Processing Protocol

After processing the blood, FEs took a loose barcode label provided in the Blood Collection Kit and affixed it to a paper manifest designed to accompany the transport tubes to LCBR. The loose barcode label matched the barcode labels on the transport tubes and the Shipping Box. FEs recorded all vacutainer tubes that were collected and identified all difficulties during blood draw or processing on the manifest as well as in the tablet. The barcode-labelled manifest was designed to be scanned on arrival at LCBR to associate it with an individual participant's transport tubes.

3.3.3 Shipment of Samples

Immediately before shipment, FEs removed two cold packs from the freezer, placed the transport tubes in a sleeve, sandwiched the transport tubes between the ice packs, enclosed the sandwich within the Styrofoam box, placed the manifest on top of the Styrofoam box, sealed the cardboard shipping box around it, put the cardboard shipping box inside the Tyvek envelope, applied a pre-printed FedEx shipping label to the envelope, carried it to a FedEx office, and handed it to a FedEx representative (*in person*) for Priority Overnight shipment to LCBR with arrival the following morning. FEs were not permitted to leave shipments at unattended FedEx drop boxes.

When overnight shipment was impossible, FEs noted this on the manifest and held unboxed transport tubes in a refrigerator approved for biological specimens or cooler with enough cold packs to keep them chilled at 4° C overnight without risk of freezing (or thawing), as is possible on wet or dry ice. The transport tubes were packaged and shipped the next day using freshly frozen cold packs.

3.3.4 Receipt of Samples at LCBR

LCBR technicians specifically trained for Add Health Wave VI received and immediately processed samples each morning. They unpacked the shipping boxes one at a time, evaluated the volume and quality of each transport tube, and entered them into a custom-made laboratory information management system (LIMS) program.

After re-centrifuging the serum samples for lipid assays at 4° C for 10 min at 30,000 g, the technicians aspirated the supernatant, discarded all remaining precipitate, transferred the aspirate to pre-labelled tubes, and placed them in a biospecimen refrigerator for archival (in 1 ml aliquots at -80° C) or assay (500 ul aliquot). The LCBR technicians entered all aliquot information into the LIMS system.

4. Assay and Internal Quality Control

4.1 Lipid Panel

4.1.1 Rationale

Cholesterol and triglycerides are lipids that travel through the blood in protein-containing particles called lipoproteins. The particles include low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and other relatively triglyceride (TG)-rich lipoproteins. Their concentrations are associated with cardiovascular disease risk^{8,9,10,11} and are targets of antihyperlipidemic therapy⁶. Total cholesterol (TC), HDL-C, and TG were therefore assayed in the serum samples that were sent to LCBR.

4.1.2 Assayed Lipid Concentrations and Internal Quality Control

All lipid assays were run on the same day of sample arrival at LCBR using an Ortho VITROS 5600 Integrated System (Ortho Clinical Diagnostics, Raritan, NJ). Serum from venous blood collected using the SST vacutainer tubes was introduced into the VITROS system by placing sample vials holding 500 µl of serum into an automatic sampling tray, after which all processes were automatically performed and results output by the VITROS system. As described below, the four lipids were assayed using colorimetric procedures that measure change in color (optical density) reflective of increases in serum lipid concentrations.

The VITROS system read barcodes on the vials to automatically determine which assays to run. In addition to the lipid procedures documented below, other assays were run from the same serum sample, including glucose, AST, ALT, and creatinine. Only the lipid assays are described below. Assay protocols for other analytes can be found in other Add Health User Guides.

4.1.2.1 Total Cholesterol [H6TC]

TC was measured using VITROS Chemistry Products CHOL slides. The Vitros CHOL slide is a multilayered, analytical element coated on a polyester support. **Figure 4** illustrates the structure and reagents of the CHOL slide.

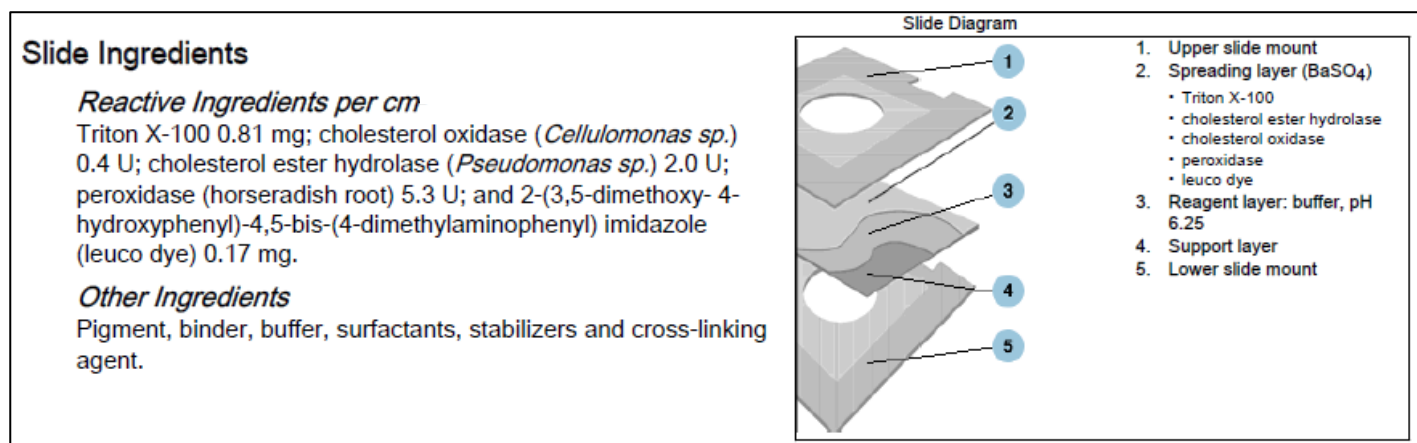


Figure 4: Ortho-Vitros CHOL Slide

A 10 µl drop of sample was deposited onto the slide and was evenly distributed by the spreading layer to the underlying layers. The presence of Triton X-100 surfactant on the spreading layer helped in dissociating the cholesterol and cholesterol esters from lipoprotein complexes present in the sample. Hydrolysis of the cholesterol esters to cholesterol was catalyzed by cholesterol ester hydrolase. Free cholesterol was then oxidized in the presence of cholesterol oxidase to form cholestenone and hydrogen peroxide. Lastly, the hydrogen peroxide oxidized a leuco dye in the presence of peroxidase to generate a colored dye¹². The density of this dye was proportional to the TC concentration present in the sample

and was measured by spectrophotometry at a wavelength of 540 nm. The specific reaction scheme is listed in **Figure 5**.

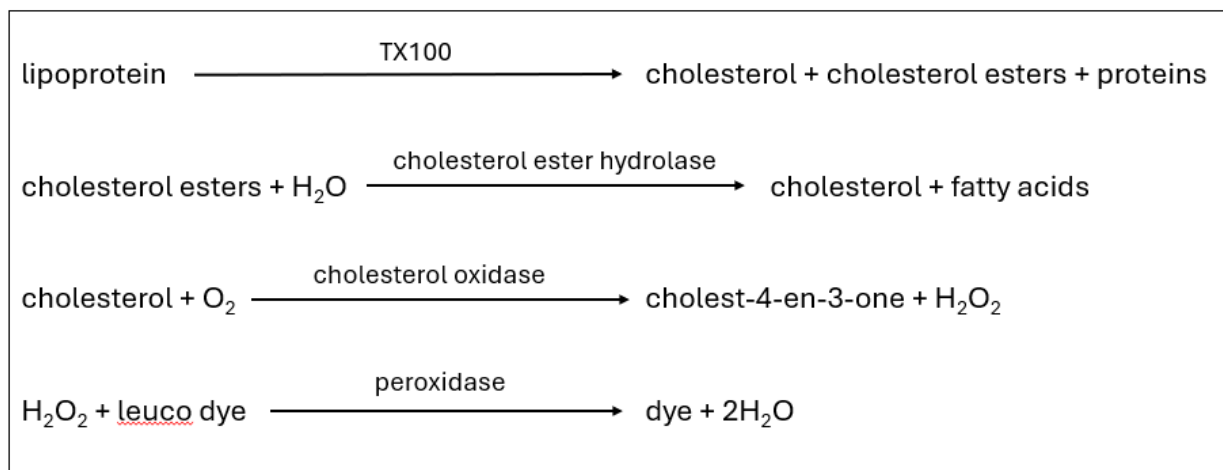


Figure 5: Total Cholesterol Reaction Assay Scheme

The intensity of the dye was measured, and corresponding TC concentration inferred at room temperature by comparing reflected light output at 540 nm to a standard curve generated using a VITROS Chemistry Products Calibrator Kit 2 (Ortho Clinical Diagnostics, Raritan, NJ). The concentrations were output to a Sunquest computer system (Sunquest Information Systems, Tucson AZ) that linked the UVMMC data with LCBR's LIMS system.

The VITROS 5600 system's dynamic reporting range of the TC assay was 50.0 - 325.0 mg/dl. When concentrations exceeded the upper limit, the VITROS system automatically diluted the samples 1:2 with a VITROS Chemistry Products FS Diluent Pack (Ortho Clinical Diagnostics, Raritan, NJ) until the concentrations were within range. Dilutions and cholesterol concentrations that accounted for the reflexive dilutions via multiplication by the dilution factor were reported simultaneously. The final TC concentrations (H6TC) ranged from 58 to 377 mg/dl.

4.1.2.2 High-Density Lipoprotein Cholesterol [H6HDL]

HDL-C was measured using VITROS Chemistry Products dHDL slides. The dHDL Slide is a multilayered analytical element coated on a polyester support. The method is based on a non-HDL precipitation method similar to one used by Burstein et al.¹³ followed by an enzymatic detection similar to that proposed by Allain et al.¹⁴ **Figure 6** illustrates the structure and reagents of the dHDL slide.

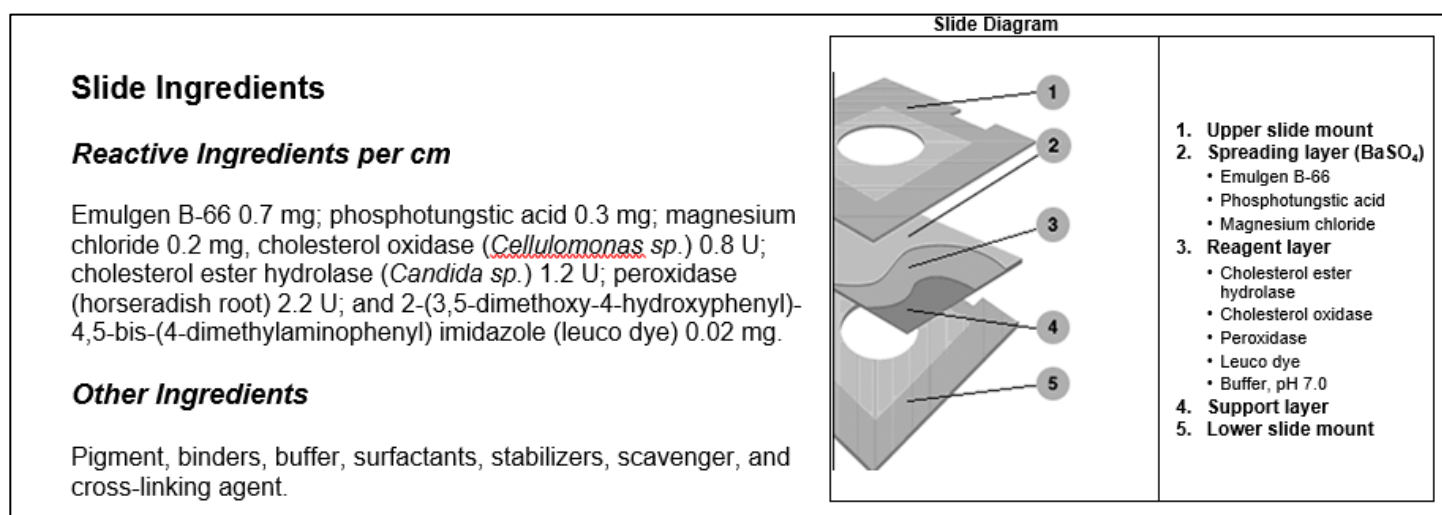


Figure 6: Ortho-Vitros dHDL Slide

A 10 μ l drop of sample was deposited on the slide and was evenly distributed by the spreading layer to the underlying layers. HDL-C was separated by the precipitation of non-HDL-C using phosphotungstic acid (PTA) and magnesium chloride (MgCl₂) in the spreading layer. Emulgen B-66, a surfactant in the spreading layer, helped in the selective dissociation of the cholesterol and cholesterol esters from the HDL-C complexes present in the sample. Hydrolysis of the HDL-C derived cholesterol ester to cholesterol was catalyzed by a selective cholesterol ester hydrolase. Free cholesterol was then oxidized in the presence of cholesterol oxidase to form cholestenone and hydrogen peroxide. Lastly, hydrogen peroxide oxidized a leuco dye in the presence of peroxidase to generate a colored dye.¹⁵ The density of the dye formed was proportional to the HDL-C concentration present in the sample and was measured by reflectance spectrophotometry at a wavelength of 670 nm. The specific reaction scheme is listed in **Figure 7**.

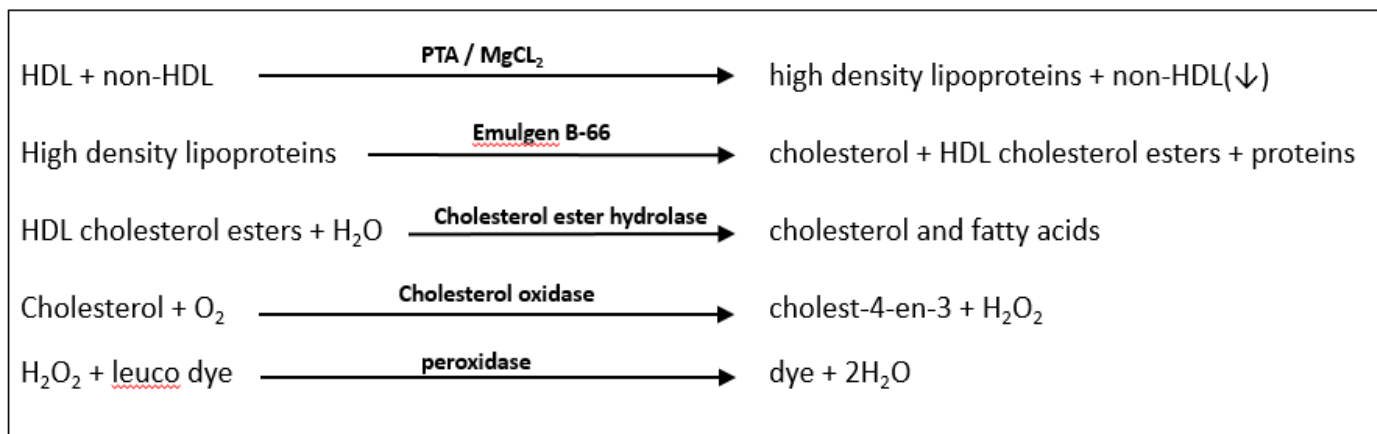


Figure 7: dHDL Assay Reaction Scheme

The serum samples were introduced into the VITROS system in the same manner as for the TC assay (see section 4.1.2.1). However, the light output for the dHDL slides was compared to a standard curve generated by the use of a VITROS Chemistry Products Calibrator Kit 25. (Ortho Clinical Diagnostics, Raritan, NJ).

The VITROS 5600 system's dynamic reporting range of the HDL-C assay was 5.0 - 110.0 mg/dl. When concentrations exceeded the upper limit, the VITROS system automatically diluted the samples 1:2 with a VITROS Chemistry Products FS Diluent Pack 2 (Ortho Clinical Diagnostics, Raritan, NJ) until the concentrations were within range. Dilutions and cholesterol concentrations that accounted for the reflexive dilutions via multiplication by the dilution factor were reported simultaneously. The final HDL-C concentrations (H6HDL) ranged from 18 to 157 mg/dl. Any values below 15 mg/dl or above 175 mg/dl were coded -9999 as implausible.

4.1.2.3 Triglycerides [H6TG]

TG were measured using VITROS Chemistry Products TRIG slides. The VITROS TRIG Slide is a multilayered, analytical element coated on a polyester support. The analysis is based on an enzymatic method as described by Spayd et al.¹⁶ **Figure 8** illustrates the structure and reagents of the TRIG slide.

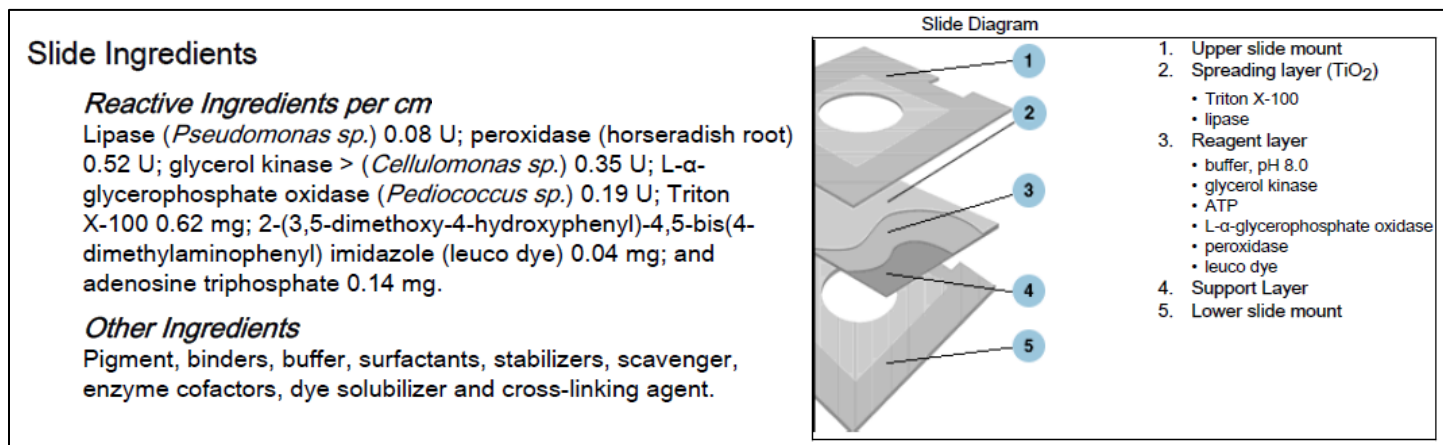


Figure 8: Ortho-Vitros TRIG Slide

A 10 μ l drop of sample was deposited on the slide and was evenly distributed by the spreading layer to the underlying layers. The presence of Triton X-100 surfactant in the spreading layer helped in dissociating TG from lipoprotein complexes present in the sample. The TG molecules were then hydrolyzed by lipase to yield glycerol and fatty acids. Glycerol diffused to the reagent layer, where it was phosphorylated by glycerol kinase in the presence of adenosine triphosphate (ATP). In the presence of L- α -glycerol-phosphate oxidase, L- α -glycerophosphate was then oxidized to dihydroxyacetone phosphate and hydrogen peroxide. Lastly, the leuco dye was oxidized by hydrogen peroxide, catalyzed by peroxidase, to produce a dye¹⁷. The density of this dye was proportional to the TG concentration present

in the sample and was measured by reflectance spectrophotometry at a wavelength of 540 nm. The specific reaction scheme is listed in **Figure 9**.

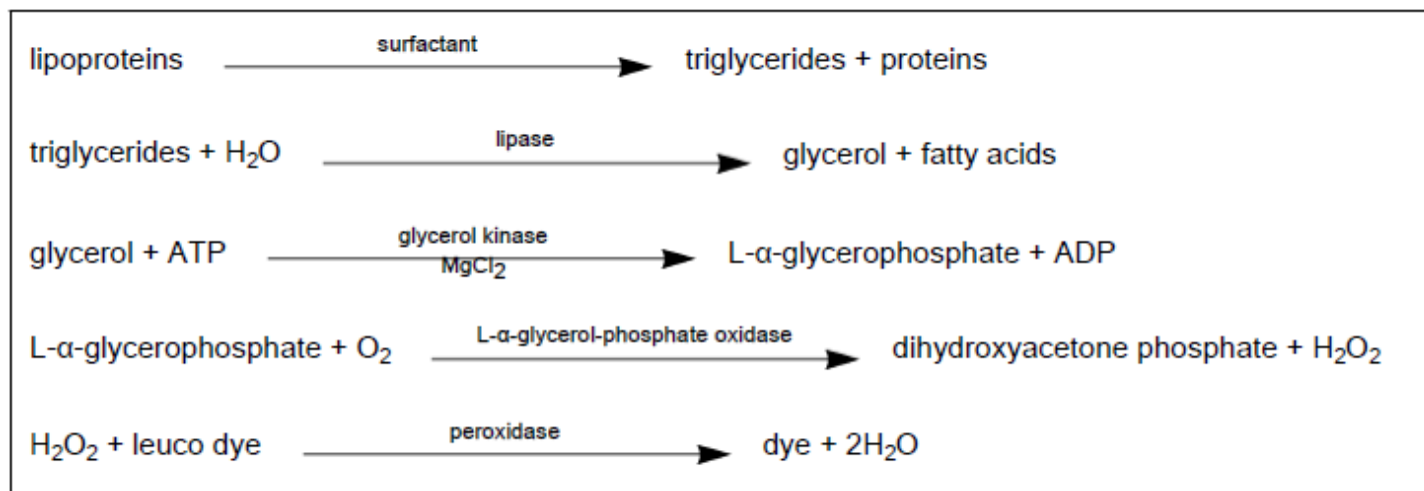


Figure 9: TRIG Assay Reaction Scheme

The serum samples were introduced into the VITROS system in the same manner as for the TC assay (see section 4.1.2.1) and the TG results were also compared to a standard curve generated by the use of a VITROS Chemistry Products Calibrator Kit 2. (Ortho Clinical Diagnostics, Raritan, NJ).

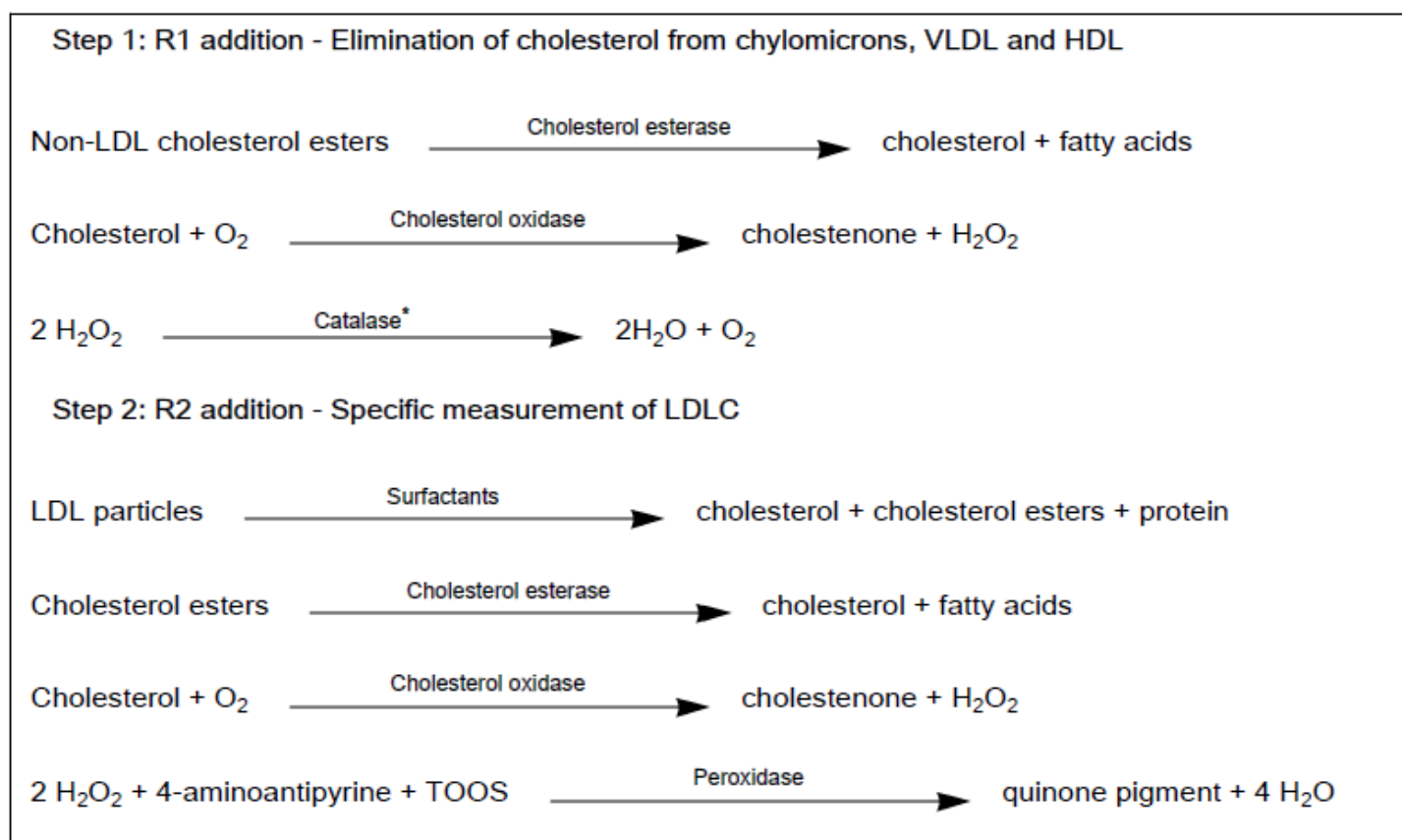
The VITROS 5600 system's dynamic reporting range of the TRIG assay was 10.0 - 525.0 mg/dl. When concentrations exceeded the upper limit, the VITROS system automatically diluted the samples 1:2 with a VITROS Chemistry Products FS Diluent Pack 2 (Ortho Clinical Diagnostics, Raritan, NJ) until the concentrations were within range. Dilutions and TG concentrations that accounted for the reflexive dilutions via multiplication by the dilution factor were reported simultaneously. The final TG concentrations (H6TG) ranged from 25 to 2238 mg/dl.

4.1.2.4 Low-Density Lipoprotein Cholesterol [H6LDL]

LDL-C concentrations were routinely computed using the Friedewald equation³ when TG < 400 mg/dl (see Section 6.1), but otherwise measured directly using the VITROS Chemistry Products dLDL Reagent in conjunction with the VITROS Chemistry Products Calibrator Kit 19 and VITROS Chemistry Products FS Calibrator 1.¹⁸ This direct method was also used if TG < 400 mg/dl and the calculated LDL-C Friedewald value was less than 20 mg/dl.

The VITROS dLDL Reagent is a dual chambered package containing stable liquid reagents that are used in a two-step reaction to quantitatively measure LDL-C. In the first step, with the addition of Reagent 1, non-LDL cholesterol (such as HDL-C, very low-density lipoprotein-cholesterol and chylomicrons) was

selectively eliminated by reaction with cholesterol esterase and cholesterol oxidase to form cholestenone and hydrogen peroxide. The peroxide generated was immediately scavenged by catalase. The addition of reagent 2 initiated the second step, in which catalase was immediately inactivated with sodium azide. Surfactants then aided in dissociation of cholesterol and cholesterol esters from LDL particles and promoted reaction with cholesterol esterase and cholesterol oxidase. The peroxide byproduct reacted with TOOS and 4-aminoantipyrine in the presence of peroxidase to form a colored quinone dye, which was measured spectrophotometrically at a wavelength of 600 nm. The specific reaction scheme is listed in **Figure 10**.



*Catalase is inhibited in the second step by sodium azide in Reagent 2

Figure 10: LDL-C Assay Reaction Scheme

The serum samples were introduced into the VITROS system in the same manner as for the TC assay (see section 4.1.2.1). However, the LDL-C results were compared to a standard curve generated by the use of a VITROS Chemistry Products Calibrator Kit 19. (Ortho Clinical Diagnostics, Raritan, NJ).

The VITROS 5600 system's dynamic reporting range of the LDL-C assay was 30.0 - 350.0 mg/dl. When concentrations exceeded the upper limit, the VITROS system automatically diluted the samples 1:2 with

a VITROS Chemistry Products FS Diluent Pack 2 (Ortho Clinical Diagnostics, Raritan, NJ) until the concentrations were within range. Dilutions and cholesterol concentrations that accounted for the reflexive dilutions via multiplication by the dilution factor were reported simultaneously. The final LDL-C concentrations (H6LDL) ranged from 20 to 307 mg/dl.

4.1.2.5 Internal Quality Control

The Ortho-VITROS system was maintained daily by cleaning machine components, replacing all reagents, and running known quality control samples (Thermo Fisher Scientific, Waltham, MA). Internal quality controls consisted of MAS OmniCORE™ quality controls. Several lots (OCR2406, OCR2511, OCR2608) were used throughout Wave VI, but all lots had similar known control values. Each lot had a low, medium, and high control and typical values for these are listed below in **Figure 11**.

OCR Control low value		OCR Control middle value		OCR Control high value	
Assay	Typical range (mg/dl)	Assay	Typical range (mg/dl)	Assay	Typical range (mg/dl)
TC	80-120	TC	140-180	TC	220-280
HDL-C	15-30	HDL-C	30-50	HDL-C	50-70
TG	80-120	TG	180-220	TG	250-310
LDL-C	N/A	LDL-C	50-80	LDL-C	80-120

Figure 11: Typical OmniCORE Control Value Levels

Values assigned to the VITROS Chemistry Products Calibrator Kit 2 for cholesterol are traceable to the Certified National Institute of Standards and Technology (NIST) Standard Reference Material (SRM®) 911. The Ortho-Clinical Diagnostics calibration laboratory uses SRM® 911 to calibrate the Centers for Disease Control (CDC) Modified Abell-Kendall method ¹⁹ to support cholesterol value assignment for VITROS Calibrator Kit 2.

Values assigned to the VITROS Chemistry Products Calibrator Kit 25 for the VITROS Chemistry Products dHDL Slides are traceable to the Cholesterol Reference Method Laboratory Network (CRMLN) Designated Comparison Method ²⁰ and NIST SRM® 911. Traceability of the CRMLN Designated Comparison Method (DCM) to the CDC Accuracy Base is maintained by each participating CRMLN Network Laboratory through the use of quarterly proficiency surveys administered by the CDC. Working Calibrators (a panel of human samples) are assigned HDL-C values by the CRMLN DCM in a CRMLN Network Laboratory for use in calibration of the Manufacturer's Standing Measurement Procedure.

Values assigned to the VITROS Chemistry Products Calibrator Kit 19 and VITROS Chemistry Products FS Calibrator Kit 1 for LDL-C are traceable to the CDC LDL-C Reference Measurement Procedure.^{21,5}

Values assigned to the VITROS Chemistry Products Calibrator Kit 2 for triglyceride are traceable to the Certified NIST (National Institute of Standards and Technology) Reference Material, SRM® (Standard Reference Material) 1951. The Ortho Clinical Diagnostics calibration laboratory uses SRM® 1951 to calibrate a glycerol phosphate oxidase (GPO) triglyceride spectrophotometric method²² to support triglyceride value assignment for VITROS Calibrator Kit 2.

In addition to the daily quality control, LCBR used two pools of samples from twenty normal donors (US Biologicals, Salem, MA) in longitudinal quality control analyses. One pool was an EDTA plasma normal donor pool (Lot #E011221). The other pool was a serum normal donor pool (Lot #S120419). LCBR periodically assayed both pools over the course of Wave V. The plasma and serum concentration mean and coefficient of variation (CV) based on those assays are tabulated in **Figure 12**. When assay concentrations exceeded acceptable parameters, the Ortho-VITROS system was investigated and repaired.

Serum Lot S120419			Plasma Lot E012221		
Assay	Mean (mg/dl)	CV(%)	Assay	Mean (mg/dl)	CV(%)
TC	181.88	3.40	TC	167.14	3.11
HDL-C	49.72	3.20	HDL-C	53.64	2.86
TG	165.16	3.31	TG	132.94	1.98

Figure 12. Quality Control Assay Values

There were no additional QC donor pools for directly measured LDL-C since this was not a standard test that the University of Vermont Hospital system ran on a regular basis.

5. External Quality Control

5.1 Reliability

Within a race/ethnicity- and sex-stratified random sample of 123 Add Health participants (89% fasting ≥ 9 hours) among whom venous blood was collected twice, on average 13.2 (95% confidence interval: 12.0-14.4) days apart, typically by the same FE and at approximately the same time of day, reliabilities of TC (mg/dl), HDL-C (mg/dl), TG (mg/dl), and LDL-C (mg/dl) were estimated as intra-class correlation coefficients (95% CI) [**Figure 13**]. The estimates suggest that the home exam venous blood collected at

Add Health Wave VI yields much more reliable measures of TC and HDL-C than the dried capillary whole blood spots collected at Wave IV, although reliability of TG is somewhat lower in this mixed group of fasting and non-fasting participants.

Measure (mg/dl)	N	ICC	95% CI
TC	123	0.90	(0.87, 0.94)
HDL-C	120	0.88	(0.83, 0.92)
TG	123	0.71	(0.63, 0.80)
LDL-C*	123	0.90	(0.86, 0.93)
*Based on the Friedewald equation when TG ≤ 400 mg/dl			

Figure 13. Reliability of Lipids

6. Constructed Measures

6.1 Constructed Lipid Concentrations and Ratio

6.1.1 Low-Density Lipoprotein Cholesterol Method of Measurement [H6LDLMM]

When TG < 400 mg/dl (default), low-density lipoprotein cholesterol was indirectly calculated from the participant-specific concentrations (mg/dl) of total cholesterol, high-density lipoprotein cholesterol, and triglycerides using the Friedewald equation, $LDL-C = TC - HDL-C - [TG \div 5]$,³ and coded as 1. When TG ≥ 400 mg/dl, or TG < 400 mg/dl and the calculated Friedewald value was < 20 mg/dl, LDL-C was directly measured (see Section 4.1.2.4) and coded as 2.

6.1.2 Non-High-Density Lipoprotein Cholesterol [H6NHDL]

Although use of the Friedewald equation³ is routine, interpretation of indirectly calculated LDL-C is difficult in the presence of post-prandial hypertriglyceridemia. The non-HDL-C concentration (mg/dl) was therefore calculated from the concentrations (mg/dl) of total cholesterol and high-density lipoprotein cholesterol, as follows: $non-HDL-C = TC - HDL-C$. This difference is reliable in the non-fasting state.⁴

6.1.3 Total to High-Density Lipoprotein Cholesterol Ratio [H6TC_HDL]

Although the ratio of total to high-density lipoprotein cholesterol concentrations (mg/dl) is neither a primary nor secondary target of antihyperlipidemic therapy,¹⁹ the ratio has been described as a simple and powerful predictor of cardiovascular disease risk^{23,24,25,26}. It was therefore calculated, as follows: $TC:HDL-C = TC / HDL-C$.

6.2 Fasting Time [H6FASTTM]

During the home exam before blood was collected, all participants were asked the following question: “At what time did you last eat or drink anything other than water, including sugar-containing candy or gum?” Responses to the question were recorded in hours and minutes and designated AM or PM. All participants were then asked, “Was that today or yesterday?” To calculate fasting times, responses to the above question were subtracted from the tablet start-of-blood collection date/time stamp. Data on fasting times outside the 0-23.9-hour range were recoded to missing among 4 participants. Values that were deemed implausible outside of 0-23.9 were assigned a code of -9999.

6.3 Fasted for Nine Hours or More [H6FASTLP]

Participants who were fasting (≥ 9 hours) and non-fasting (< 9 hours) at the time of blood collection were identified (1,0) based on NCEP recommendations.⁵

6.4 Lipid Classifications

6.4.1 According to Decile Rank

To facilitate comparison with Wave IV lipid deciles, all lipid concentrations and the TC:HDL-C ratio were ordered and assigned decile ranks:

- Total cholesterol decile (H6TCDEC)
- High-density lipoprotein cholesterol decile (H6HDLDEC)
- Triglycerides decile (H6TGDEC)
- Low-density lipoprotein cholesterol decile (H6LDLDEC)
- Non-high-density lipoprotein cholesterol decile (H6NHDEC)
- Total to high-density lipoprotein cholesterol ratio decile (H6TCHDEC)

6.4.2 According to the National Cholesterol Education Program (NCEP) / Adult Treatment Panel (ATP) III guidelines

The following fasting lipid classifications were constructed based on NCEP/ATP III guidelines⁶ as described in **Figures 14-17**:

Classification [H6CTC]	Fasting TC (mg/dl)	NCEP/ATP III Class
1	< 200	Desirable
2	200 - 239	Borderline high
3	≥ 240	High

Figure 14. Total Cholesterol

Classification [H6CHDL]	Fasting HDL-C (mg/dl)	NCEP/ATP III Class
1	< 40	Low
2	40 – 59	Optimal
3	≥ 60	High

Figure 15. High-Density Lipoprotein Cholesterol

Classification [H6C1TG]	Fasting TG (mg/dl)	NCEP/ATP III Class
1	< 150	Normal
2	150 - 199	Borderline high
3	200 - 499	High
4	≥ 500	Very high

Figure 16. Triglycerides

Classification [H6C1LDL]	Fasting LDL-C (mg/dl)	NCEP/ATP III Class
1	< 100	Optimal
2	100 - 129	Near optimal
3	130 - 159	Borderline high
4	160 - 189	High
5	≥ 190	Very high

Figure 17. Low-Density Lipoprotein Cholesterol

6.4.3 According to the American College of Cardiology (ACC) / American Heart Association (AHA) guidelines

The following lipid classifications were constructed based on ACC/AHA guidelines⁷ as described in **Figures 18-19**:

Classification [H6C2TG]	TG (mg/dl)	ACC/AHA Class
	Fasting or Non-Fasting	
1	< 175	Neither moderate nor severe hypertriglyceridemia
2	175 - 499	Moderate hypertriglyceridemia
3	≥ 500	Severe hypertriglyceridemia fasting
4	≥ 500	Severe hypertriglyceridemia non-fasting
5	≥ 500	Severe hypertriglyceridemia fasting unknown

Figure 18. Total Glycerides

Classification [H6C2LDL]	LDL-C (mg/dl)	ACC/AHA Class
	Fasting or Non-Fasting	
1	< 160	Neither moderate nor severe hypercholesterolemia
2	160 - 189	Moderate hypercholesterolemia
3	≥ 190	Severe hypercholesterolemia

Figure 19. Low-Density Lipoprotein Cholesterol

6.5 Antihyperlipidemic Medication Use [H6C_MED2]

Use of a prescription medication in the past four weeks in one or more of the therapeutic classes listed in **Figure 20** was assigned a value of 1. Non-use of a prescription medication in the past four weeks in one of the therapeutic classes listed below was assigned a value of 0.

Class	Label	Variable
358-019-***	Antihyperlipidemic agents	H6C_MED2
358-019-173	HMG-CoA reductase inhibitors	
358-019-174	Miscellaneous antihyperlipidemic agents	
358-019-241	Fibric acid derivatives	

358-019-252	Bile acid sequestrants	
358-019-316	Cholesterol absorption inhibitors	
358-019-317	Antihyperlipidemic combinations	
358-019-484	PCSK9 inhibitors	

Figure 20. Antihyperlipidemic Medications

Therapeutically classified use of prescription medication in particular classes may confound biomarker-based estimates of disease prevalence or risk. For example, use of antihyperlipidemic medications may confound lipid-based estimates of hyperlipidemia prevalence or cardiovascular disease risk. However, the (1,0) classifications should be used cautiously in the investigation or control of potential confounding, because selection biases often threaten the study of non-randomized medication exposures.^{27,28}

6.6 Joint Classification of Hyperlipidemia [H6HLIPJC]

Participants were flagged as having evidence of hyperlipidemia if they met **at least one** of the following criteria in **Figure 21**. This joint classification variable incorporates the ACC/AHA classifications of TG and LDL-C concentrations:

Criterion (≥ 1 of the following must be true)	Variable & Value
Self-reported history of high cholesterol or triglycerides	H6CQ47F = 1
Antihyperlipidemic medication use in the past 4 weeks	H6C_MED2 = 1
Fasting triglycerides ≥ 500 mg/dl	H6C2TG = 3
Low-density lipoprotein cholesterol ≥ 190 mg/dl	H6C2LDL = 3

Figure 21. Criteria Used to Identify Hyperlipidemia

6.7 Joint Classification of Hyperlipidemia as at Wave IV [H6HLPJC4]

Participants were flagged as having evidence of hyperlipidemia if they met **at least one** of the following criteria in **Figure 22**:

Criterion (≥ 1 of the following must be true)	Variable & Value
Self-reported history of high cholesterol or triglycerides	H6CQ47F = 1
Antihyperlipidemic medication use in the past 4 weeks	H6C_MED2 = 1

Figure 22. Criteria Used to Identify Hyperlipidemia as at Wave IV

7. The Lipids Data File (blipids6.sas7bdat)

7.1. Structure

The structure of the disseminated lipids data file is flat. This means that it is a participant-level data file, where each participant has one and only one record. The participant's identifying number (the AID variable) will appear in the data file only once.

7.2. Contents

The lipids data file includes the variables below, which are described in the corresponding codebook documentation that also contains frequencies.

<u>Variable Name</u>	<u>Variable Description</u>
AID	Participant Identifier
H6TC	Total cholesterol (TC, mg/dl)
H6HDL	HDL cholesterol (HDL-C, mg/dl)
H6TG	Triglycerides (TG, mg/dl)
H6LDL	LDL cholesterol (LDL-C, mg/dl)
H6LDLMM	LDL-C Method of measurement
H6NHDL	Non-HDL cholesterol (non-HDL-C, mg/dl)
H6TC_HDL	TC to HDL-C ratio (TC:HDL-C)
H6FASTTM	Hours since last ate
H6FASTLP	Fasted for 9+ hours
H6TCDEC	Total cholesterol (TC) decile
H6HDLDEC	HDL cholesterol (HDL-C) decile
H6TGDEC	Triglycerides (TG) decile
H6LDLDEC	LDL cholesterol (LDL-C) decile
H6NHDEC	Non-HDL cholesterol (non-HDL-C) decile
H6TCHDEC	TC to HDL-C ratio (TC:HDL-C) decile
H6CTC	Classification of TC (NCEP/ATP III)
H6CHDL	Classification of HDL-C (NCEP/ATP III)
H6C1TG	Classification of TG (NCEP/ATP III)
H6C1LDL	Classification of LDL-C (NCEP/ATP III)
H6C2TG	Classification of TG (AHA/ACC)
H6C2LDL	Classification of LDL-C(AHA/ACC)
H6C_MED2	Antihyperlipidemic medication use in past 4 weeks
H6CQ47F	CQ47f: Ever diagnosed with high cholesterol, triglycerides, or lipids
H6HLIPJC	Hyperlipidemia joint classification
H6HLPJC4	Hyperlipidemia joint classification (Wave IV)

7.3. Use

Despite attempts to harmonize methods across Waves IV-VI, important inter-Wave differences in protocols, biospecimens, assays, and data quality exist, as grey-highlighted here and above. Their existence suggests that the measures of lipids described in Sections 1-6 may not be readily comparable from wave to wave. Caution should therefore be exercised when leveraging repeated measures of lipids from Wave IV-VI, whether they are primary measures or constructed classifications. Indeed, the merit of pre-analytical z-transformation or quantile-based classification of Wave IV-VI biomarkers (lipids) and the potential pitfall otherwise associated with equating values in their original units (mg/dl) across visits should be carefully considered before using these data.

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