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National Longitudinal Study of Adolescent to Adult Health (Add Health), 1994-2018 [Public Use]

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Wave V: Biomarkers, Lipids User Guide

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2020

Add Health Wave V Documentation

Lipids

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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 V home exam. Whenever possible, data collection and methods in Wave V mirrored those of Wave IV to ensure comparability of data between waves, although important inter-Wave differences 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:

- Anthropometrics
- Cardiovascular Measures
- Medication Use – Home Exam
- Baroreflex Sensitivity & Hemodynamic Recovery
- Glucose Homeostasis
- Inflammation and Immune Function
- Renal Function

Acknowledgement

This research uses data from Add Health, a program project directed by Kathleen Mullan Harris and designed by J. Richard Udry, Peter S. Bearman, and Kathleen Mullan Harris at the University of North Carolina at Chapel Hill, and funded by grant P01-HD31921 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development, with cooperative funding from 23 other federal agencies and foundations. We gratefully acknowledge Carolyn Halpern, Nancy Dole, Joyce Tabor, and Sarah Dean for their dedication to the quality of the Add Health data in this document. Information on how to obtain the Add Health data files is available on the Add Health website (https://addhealth.cpc.unc.edu/).

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Add Health is a program project directed by Kathleen Mullan Harris and designed by J. Richard Udry, Peter S. Bearman, and Kathleen Mullan Harris at the University of North Carolina at Chapel Hill, and funded by grant P01-HD31921 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development, with cooperative funding from 23 other federal agencies and foundations. Information on how to obtain the Add Health data files is available on the Add Health website (http://www.cpc.unc.edu/addhealth).

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1. Introduction

Wave IV lipid concentrations were measured using dried blood spots collected using capillary finger prick[1]. In contrast, Wave V lipid concentrations were based on venous blood collected via phlebotomy. 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 V data include nineteen constructed measures designed to facilitate analysis and interpretation of lipid concentrations:

Constructed Lipid Concentrations and Ratio

- Low-density lipoprotein cholesterol[2] (LDL-C, mg/dl), if TG ≤ 400 mg/dl
- Non-high-density lipoprotein cholesterol[3] (non-HDL-C, mg/dl)
- Total to high-density lipoprotein cholesterol ratio[4] (TC:HDL-C)

Lipid Classifications

- According to decile rank
- According to NCEP/ATP III guidelines[5]
- According to AHA/ACC guidelines[6]

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

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2. General Overview of Data Collection

All Wave V venous blood samples were collected during home exams performed by FEs from two Add Health data collection partners: Examination Management Services, Inc. (2016–2017) and Hooper Holmes, Inc. (2018–2019). All FEs were trained and certified using a custom program specific to the Add Health protocol. FEs used a 7" Samsung Galaxy Tab 4 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 respondents. 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 respondent. All respondents 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, 91.8% of the respondents agreed to and completed the blood draw. Of the remainder, 6.5% refused, 1.3% agreed but the blood draw was unsuccessful, and < 1% had exams terminated before the blood draw (see the blood draw status variable **H5BLOOD** in the *bdemo5* 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^{o} 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 respondent. 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 am 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 a pre-specified panel of metabolic, hematologic, inflammatory, immune, and renal biomarkers, 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.

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3.2 Equipment

Before exams, FEs were shipped a Visit Supply Kit (Exhibit 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) 6 ml sodium fluoride/potassium oxalate (NaFl/KOx)-containing vacutainer tube, if needed for the glucose sub-study (see Section 3.3.1)
- (1) 3- or 4- 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 the vacutainer and transport tubes. As of February 2018, their 3 ml EDTA vacutainer tube (Cat #367835) was no longer available, so Add Health switched to the 4 ml EDTA vacutainer tube (Cat #367844).

Exhibit 1. Visit Supply and Blood Collection Kits

FEs were responsible for providing ancillary materials for each home exam, including but not limited to a

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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 respondents were comfortable giving blood, respondents 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. Scanning it automatically captured a unique, eight-digit code, thereby linking the respondent to the transport tubes / labels within it, the corresponding ODK questionnaire data, and ultimately to LCBR results.

Following standard phlebotomy protocols, FEs asked respondents 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 respondents had objections, venipuncture was performed on the best potential vein and whole blood was collected, as summarized below:

- Put on nitrile gloves.
- Have the respondent 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 respondent 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 Exhibit 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 respondent becomes uncomfortable. The respondent may open his/her fist once blood flow is established.

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- 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 respondent 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 respondent to sit quietly for a few minutes. Due to a fasting blood draw encourage the respondent 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.

Either 5 or 6 tubes of blood were collected per respondent, depending on eligibility for a separate glucose sub-study (see Measures of Glucose Homeostasis User Guide, Section 4.1.2.1). Collection order, tube type, and processing information are listed below (Exhibit 2).

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Order	Tube Type	Centrifuged	Resultant supernatant	Resultant precipitate	Use
1	8.5 ml SST	Yes	Serum	Discarded	Assays: glucose, total cholesterol, high- & low-density lipoprotein-cholesterol, triglycerides, high sensitivity C reactive protein, creatinine & cystatin C
2	10 ml EDTA	Yes	Plasma	RBC/buffy coat	Archival: for future use
3	3 or 4 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	6 ml NaFl/KOx	Yes	Plasma	Discarded	Assay: glucose sub-study
6	10 ml PAXgene	No	N/A	N/A	Archival: for future use

Exhibit 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^{o} C (on ice or frozen cold packs) in the interim.

All FEs centrifuged specific blood collection tubes, including the 8.5 ml SST, 10 ml EDTA, and when collected, the 6 ml NaFl/KOx vacutainer tubes. The 3-4 ml EDTA vacutainer tube used for the HbA1c assay was *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 and (separately, when collected) plasma from the NaFl/KOx vacutainer 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, a blue cap identified transport tubes containing plasma from the 10 ml EDTA vacutainer tube, and a white cap identified the transport tube containing plasma from the Na/Fl/KOx tube. Transport tubes were chilled at 4^{o} C (on ice or frozen cold packs) until packaged for shipment to LCBR. Exhibit 3 demonstrates the complete blood processing protocol.

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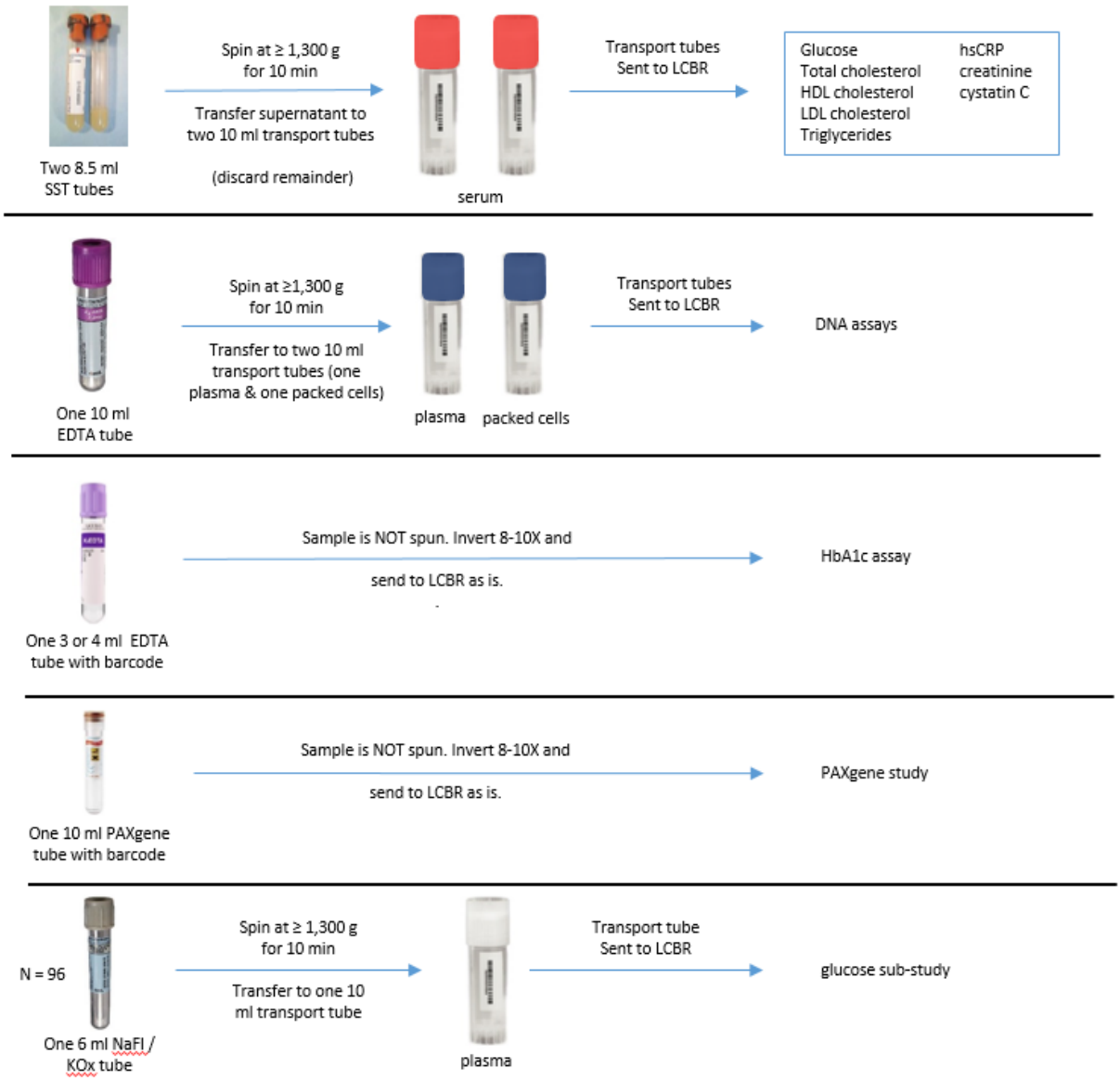

Exhibit 3. Blood Processing Protocol

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 respondent's transport tubes.

3.3.3 Shipment of Samples

Immediately before shipment, FEs removed two cold packs from the freezer, sandwiched the transport tubes between them, enclosed the sandwich within the Styrofoam Box, placed the manifest on top of

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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^{o} 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 V 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[7,8,9,10] and are targets of antihyperlipidemic therapy[5]. 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, creatinine, hsCRP, and Cystatin C. 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 [H5TC]

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

Slide Ingredients

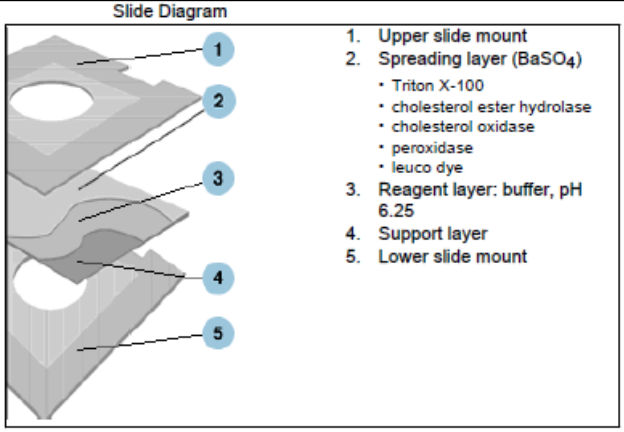
Reactive Ingredients per cm

Triton X-100 0.81 mg; cholesterol oxidase (*Cellulomonas sp.*) 0.4 U; cholesterol ester hydrolase (*Pseudomonas sp.*) 2.0 U; peroxidase (horseradish root) 5.3 U; and 2-(3,5-dimethoxy- 4-hydroxyphenyl)-4,5-bis-(4-dimethylaminophenyl) imidazole (leuco dye) 0.17 mg.

Other Ingredients

Pigment, binder, buffer, surfactants, stabilizers and cross-linking agent.

Slide Diagram



1. Upper slide mount
2. Spreading layer ($BaSO_4$)
   - Triton X-100
   - cholesterol ester hydrolase
   - cholesterol oxidase
   - peroxidase
   - leuco dye
3. Reagent layer: buffer, pH 6.25
4. Support layer
5. Lower slide mount

Exhibit 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 dye[11]. 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 Exhibit 5.

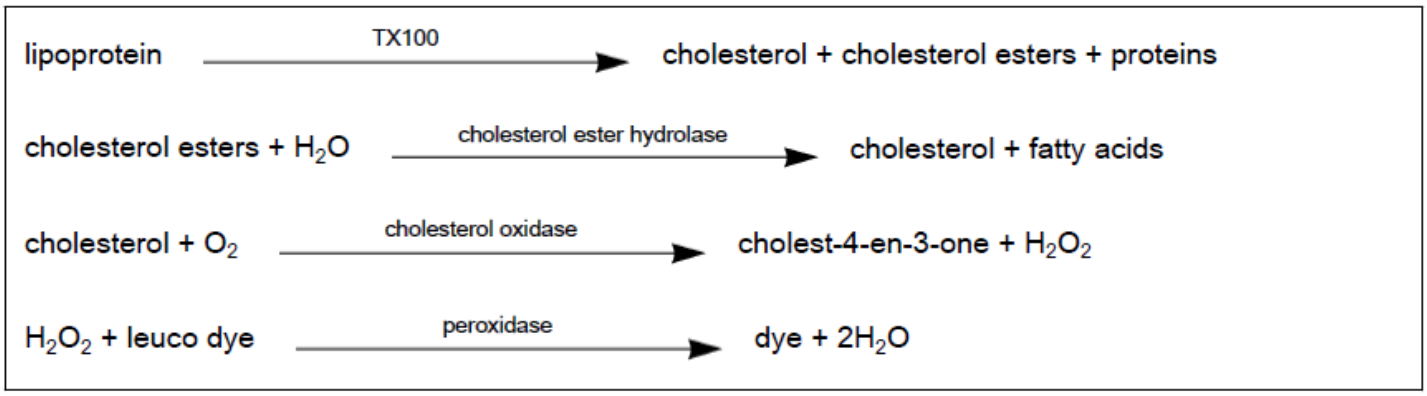


Exhibit 5: Total Cholesterol Reaction Assay Scheme

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 (**H5TC)** ranged from 62 to 424 mg/dl.

4.1.2.2 High-Density Lipoprotein Cholesterol [H5HDL]

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.[12] followed by an enzymatic detection similar to that proposed by Allain et al.[13] Exhibit 6 illustrates the structure and reagents of the dHDL slide.

Slide Ingredients

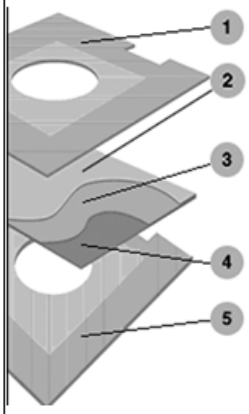
Reactive Ingredients per cm

Emulgen B-66 0.7 mg; phosphotungstic acid 0.3 mg; magnesium chloride 0.2 mg, cholesterol oxidase (*Cellulomonas sp.*) 0.8 U; cholesterol ester hydrolase (*Candida sp.*) 1.2 U; peroxidase (horseradish root) 2.2 U; and 2-(3,5-dimethoxy-4-hydroxyphenyl)-4,5-bis-(4-dimethylaminophenyl) imidazole (leuco dye) 0.02 mg.

Other Ingredients

Pigment, binders, buffer, surfactants, stabilizers, scavenger, and cross-linking agent.

Slide Diagram



1. Upper slide mount
2. Spreading layer ($BaSO_4$)
   - Emulgen B-66
   - Phosphotungstic acid
   - Magnesium chloride
3. Reagent layer
   - Cholesterol ester hydrolase
   - Cholesterol oxidase
   - Peroxidase
   - Leuco dye
   - Buffer, pH 7.0
4. Support layer
5. Lower slide mount

Exhibit 6: Ortho-Vitros dHDL slide

A 10 µl 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 ($MgCl_2$) 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.[14] 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 Exhibit 7.

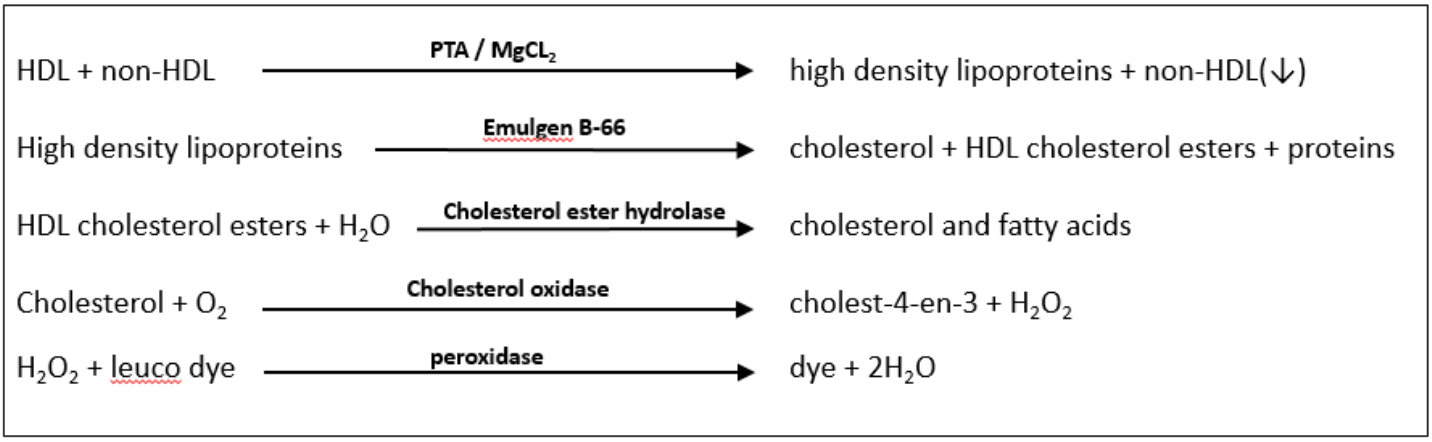


Exhibit 7: dHDL Assay Reaction Scheme

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

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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 (**H5HDL)** ranged from 17 to 156 mg/dl.

4.1.2.3 Triglycerides [H5TG]

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.[15] Exhibit 8 illustrates the structure and reagents of the TRIG slide.

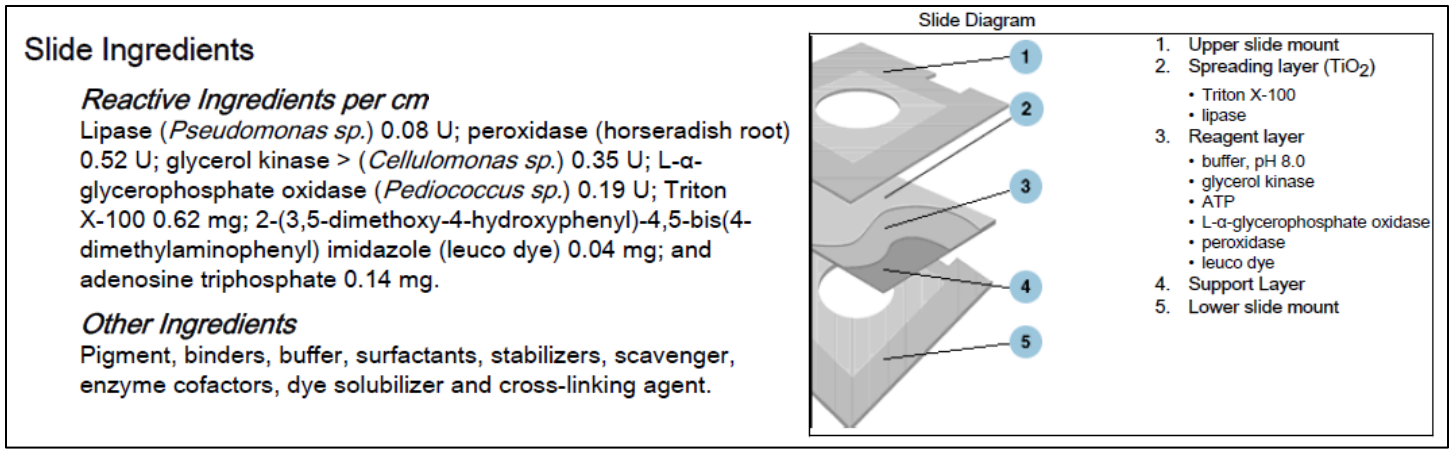

Exhibit 8: Ortho-Vitros TRIG slide

A 10 µl 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 dye[16]. 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 Exhibit 9.

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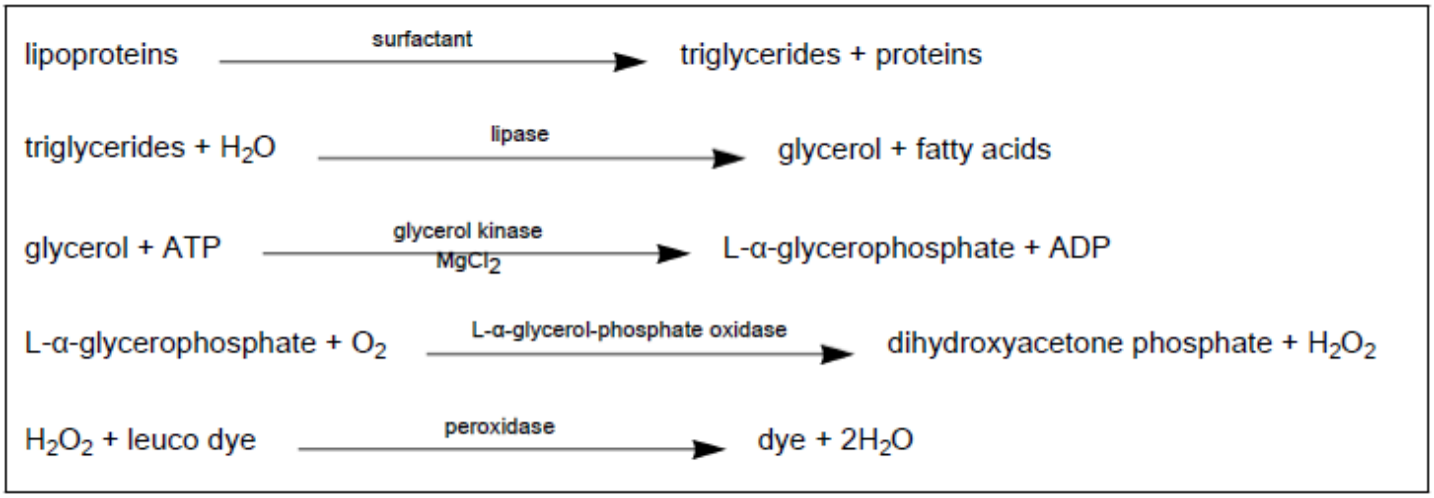

Exhibit 9: TRIG Assay Reaction Scheme

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 (**H5TG)** ranged from 22 to 1604 mg/dl.

4.1.2.4 Low-Density Lipoprotein Cholesterol [H5LDL]

LDL-C concentrations were routinely computed using the Friedewald equation[2] 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.[17]

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) is selectively eliminated by reaction with cholesterol esterase and cholesterol oxidase to form cholestenone and hydrogen peroxide. The peroxide generated is immediately scavenged by catalase. The addition of reagent 2 initiates the second step, in which catalase is immediately inactivated with sodium azide. Surfactants then aid in dissociation of cholesterol and cholesterol esters from LDL particles and promote reaction with cholesterol esterase and cholesterol oxidase. The peroxide byproduct reacts with TOOS and 4-aminoantipyearine in the presence of peroxidase to form a colored

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quinone dye, which is measured spectrophotometrically at a wavelength of 600 nm. The specific reaction scheme is listed in Exhibit 10.

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

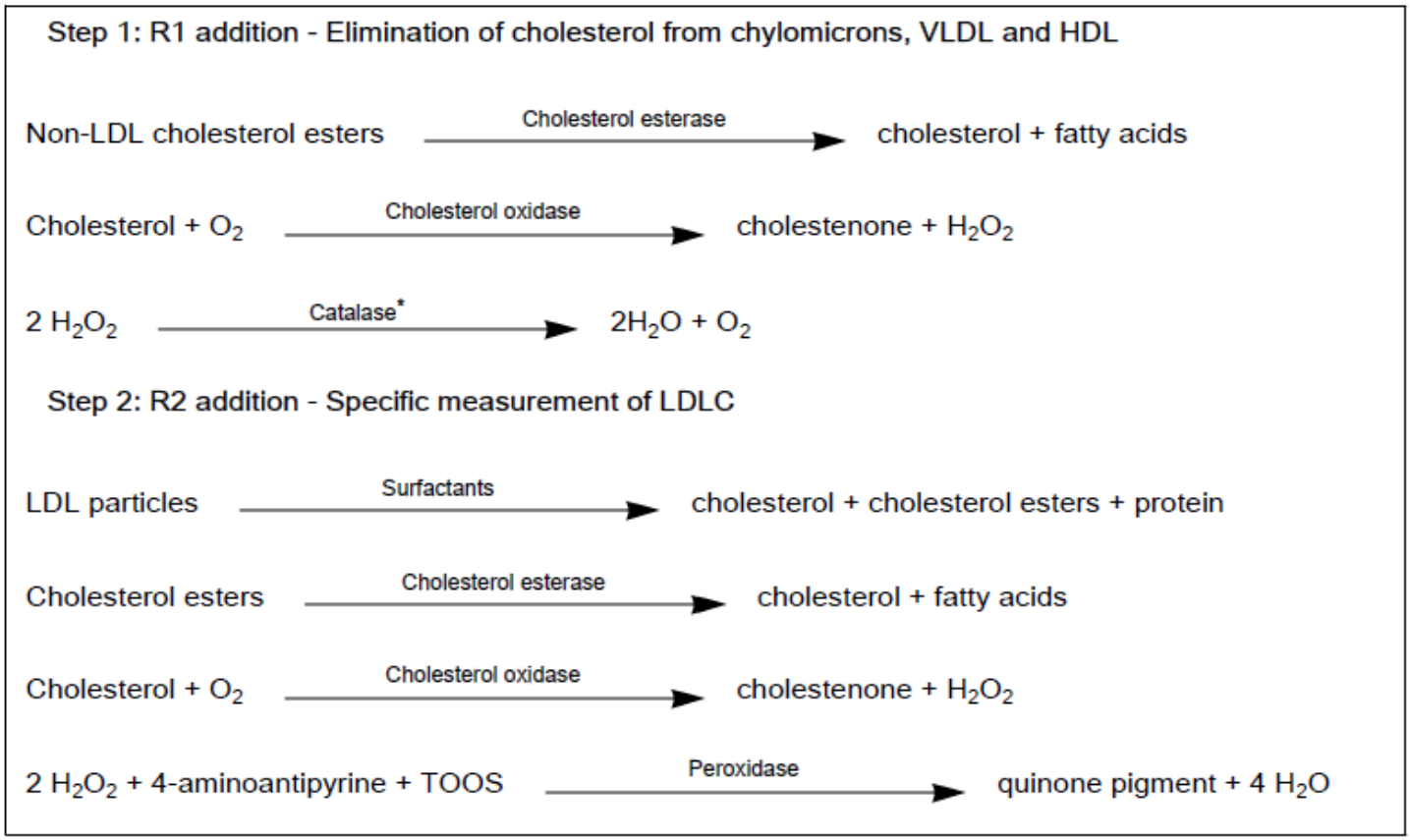

Exhibit 10: LDL-C Assay Reaction Scheme

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 (**H5LDL)** ranged from 9 to 332 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 (Ortho Clinical Diagnostics, Raritan, NJ). Internal quality

controls consisted of VITROS Performance Verifier I, and Vitros Performance Verifier 2. Typical values for these are listed below in Exhibit 11.

Performance Verifier I		Performance Verifier II	
Assay	**Typical range (mg/dl)**	**Assay**	**Typical range (mg/dl)**
TC	145-180	TC	225-270
HDL-C	45-60	HDL-C	60-75
TG	100-150	TG	210-290
LDL-C	60-85	LDL-C	100-130

Exhibit 11: Typical Performance Verifier 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 [18] 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 [19] 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.[20,4]

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 #E050115). The other pool was a serum normal donor pool (Lot #S042715). 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 Exhibit 12. When assay concentrations exceeded acceptable parameters, the Ortho-VITROS system was investigated and repaired.

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Serum Lot S042715			Plasma Lot E051515		
Assay	**Mean (mg/dl)**	**CV**	**Assay**	**Mean (mg/dl)**	**CV**
TC	138	3.02	TC	146	3.94
HDL-C	42	3.20	HDL-C	58	3.77
TG	122	4.35	TG	88	3.71

Exhibit 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 103 Add Health respondents (81% fasting ≥ 8 hours) among whom venous blood was collected twice, on average 14.3 (95% confidence interval: 13.0-15.5) 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) [Exhibit 13]. The estimates suggest that the home exam venous blood collected at Add Health Wave V 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 respondents.

Measure (mg/dL)	N	ICC	95% CI
TC	103	0.88	(0.84, 0.92)
HDL-C	103	0.95	(0.94, 0.97)
TG	103	0.63	(0.51, 0.74)
LDL-C*	103	0.86	(0.81, 0.91)
***Based on the Friedewald equation when TG ≤ 400 mg/dl**			

Exhibit 13. Reliability of Lipids

6. Constructed Measures

6.1 Constructed Lipid Concentrations and Ratio

6.1.1 Low-Density Lipoprotein Cholesterol Method of Measurement [H5LDLMM]

When TG < 400 mg/dl (default), low-density lipoprotein cholesterol was indirectly calculated from the

respondent-specific concentrations (mg/dl) of total cholesterol, high-density lipoprotein cholesterol, and triglycerides using the Friedewald equation, LDL-C = TC – HDL-C – [TG ÷ 5],[2] and coded as 1. When TG ≥ 400 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 [**H5NHDL**]

Although use of the Friedewald equation[2] 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.[3]

6.1.3 Total to High-Density Lipoprotein Cholesterol Ratio [**H5TC_HDL**]

Although the ratio of total to high-density lipoprotein cholesterol concentrations (mg/dl) is neither a primary nor secondary target of antihyperlipidemic therapy,[18] the ratio has been described as a simple and powerful predictor of cardiovascular disease risk [21,22,23,24]. It was therefore calculated, as follows: TC:HDL-C = TC / HDL-C.

6.2 Fasting Time [**H5FASTTM**]

During the home exam before blood was collected, all respondents 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 respondents 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 60 respondents. Values that were deemed implausible (≥ 24 hours) were assigned a code of 999, while negative values that were due to unrealistic computer date/time stamps were assigned a code of -999.

6.3 Fasted for Nine Hours or More **[H5FASTLP]**

Respondents who were fasting (≥ 9 hours) and non-fasting (< 9 hours) at the time of blood collection were identified (1,0) based on NCEP recommendations.[4]

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 (**H5TCDEC**)
- High-density lipoprotein cholesterol decile (**H5HDLDEC**)

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- Triglycerides decile (**H5TGDEC**)
- Low-density lipoprotein cholesterol decile (**H5LDLDEC**)
- Non-high-density lipoprotein cholesterol decile (**H5NHDEC**)
- Total to high-density lipoprotein cholesterol ratio decile (**H5TCHDEC**)

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 guidelines[5] as described in Exhibits 14-17:

Classification [H5CTC]	Fasting TC (mg/dl)	NCEP/ATP III Class
1	< 200	Desirable
2	200 - 239	Borderline high
3	≥ 240	High

Exhibit 14. Total Cholesterol

Classification [H5CHDL]	Fasting HDL-C (mg/dl)	NCEP/ATP III Class
1	< 40	Low
2	40 – 59	Optimal
3	≥ 60	High

Exhibit 15. High-Density Lipoprotein Cholesterol

Classification [H5C1TG]	Fasting TG (mg/dl)	NCEP/ATP III Class
1	< 150	Normal
2	150 - 199	Borderline high
3	200 - 499	High
4	≥ 500	Very high

Exhibit 16. Triglycerides

Classification [H5C1LDL]	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

Exhibit 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 guidelines[6] as described in Exhibits 18-19:

Classification [H5C2TG]	TG (mg/dl)	ACC/AHA Class
	Fasting or Non-Fasting	
1	< 175	Neither moderate nor severe hypertriglyceridemia
2	175 - 499	Moderate hypertriglyceridemia
	Fasting	
3	≥ 500	Severe hypertriglyceridemia

Exhibit 18. Total Glycerides

Classification [H5C2LDL]	Fasting or Non-Fasting LDL-C (mg/dl)	ACC/AHA Class
1	< 160	Neither moderate nor severe hypercholesterolemia
2	160 - 189	Moderate hypercholesterolemia
3	≥ 190	Severe hypercholesterolemia

Exhibit 19. Low-Density Lipoprotein Cholesterol

6.5 Antihyperlipidemic Medication Use **[H5C_MED2]**

Use of a prescription medication in the past four weeks in one or more of the therapeutic classes listed

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in Exhibit 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
358-019-173	HMG-CoA reductase inhibitors	H5C_MED2
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	

Exhibit 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.

6.6 Joint Classification of Hyperlipidemia [H5HLIPJC]

Respondents were flagged as having evidence of hyperlipidemia if they met **at least one** of the following criteria in Exhibit 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	H5Q045F = 1
Antihyperlipidemic medication use in the past 4 weeks	H5C_MED2 = 1
Fasting triglycerides ≥ 500 mg/dl	H5C2TG = 3
Low-density lipoprotein cholesterol ≥ 190 mg/dl	H5C2LDL = 3

Exhibit 21. Criteria Used to Identify Hyperlipidemia

6.7 Joint Classification of Hyperlipidemia as at Wave IV [H5HLPJC4]

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

Criterion (≥ 1 of the following must be true)	Variable & Value
Self-reported history of high cholesterol or triglycerides	H5Q045F = 1
Antihyperlipidemic medication use in the past 4 weeks	H5C_MED2 = 1

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

7. The Lipids Data File (blipids5.xpt)

7.1. Structure

The structure of the disseminated lipids data file is flat. This means that it is a respondent-level data file, where each respondent has one and only one record. The respondent'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.

Variable Name	**Variable Description**
AID	Respondent Identifier
H5TC	Total cholesterol concentration (mg/dl)
H5TCDEC	Total cholesterol concentration as a decile
H5HDL	HDL cholesterol concentration (mg/dl)
H5HDLDEC	HDL cholesterol concentration as a decile
H5TG	Triglycerides concentration (mg/dl)
H5TGDEC	Triglycerides concentration as a decile
H5LDL	LDL cholesterol concentration (mg/dl)
H5LDLDEC	LDL cholesterol concentration as a decile
H5LDLMM	Method of measurement for LDL-C
H5NHDL	Non-high-density lipoprotein cholesterol concentration (mg/dl)
H5NHDEC	Non-high-density lipoprotein cholesterol concentration as a decile
H5TC_HDL	Total cholesterol to HDL cholesterol ratio (TC:HDL-C)
H5TCHDEC	Total cholesterol to HDL cholesterol ratio as a decile
H5FASTTM	Number of hours since last ate
H5FASTLP	Flag indicating fasting for nine hours or more
H5CTC	Fasting TC classification (NCEP/ATP III)
H5CHDL	Fasting HDL-C classification (NCEP/ATP III)
H5C1TG	Fasting TG classification (NCEP/ATP III)
H5C1LDL	Fasting LDL-C classification (NCEP/ATP III)
H5C2TG	TG classification (AHA/ACC)

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H5C2LDL	LDL-C classification (AHA/ACC)
H5Q045F	Q045f Ever diagnosed with high cholesterol or triglycerides
H5C_MED2	Flag indicating antihyperlipidemic medication use
H5HLIPJC	Hyperlipidemia joint classification
H5HLPJC4	Hyperlipidemia joint classification (Wave IV version)

7.3. Use

Despite attempts to harmonize methods across Waves IV and V, 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-V, whether they are primary measures or constructed classifications. Indeed, the merit of pre-analytical z-transformation or quantile-based classification of Wave IV-V 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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