6. Laboratory Procedure

6.1 PROCEDURE FOR BLOOD DRAWING

<table>
<thead>
<tr>
<th>Have the following tubes labelled and ready in ice bucket</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting Sample</td>
</tr>
<tr>
<td>* Three 10-ml Lavender-top tubes</td>
</tr>
<tr>
<td>* One 3-ml Gray-top tube</td>
</tr>
<tr>
<td>2-hr Sample</td>
</tr>
<tr>
<td>* One 5-ml Lavender-top tube</td>
</tr>
<tr>
<td>* One 3-ml Gray-top tube</td>
</tr>
<tr>
<td>Other Supplies</td>
</tr>
<tr>
<td>* tourniquet</td>
</tr>
<tr>
<td>* vaccutainer sleeve</td>
</tr>
<tr>
<td>* alcohol pads</td>
</tr>
<tr>
<td>* vaccutainer needle [21G]</td>
</tr>
<tr>
<td>* 2x2 gauze pads</td>
</tr>
<tr>
<td>(multiple sample)</td>
</tr>
<tr>
<td>* bandaids</td>
</tr>
<tr>
<td>* urine collection cups</td>
</tr>
</tbody>
</table>

Note:
Participant exempted from the GTT will also have 5-ml Lavender top tube drawn at fasting.

6.1.1 Accuchek Procedure

1) Obtain an Accuchek reading from a drop of blood obtained by finger prick. (Using the blood from the venipuncture procedure below will not provide comparable results since there is a difference between capillary blood (fingerstick) and venous blood values.)

2) See Accuchek procedure for calibrating the meter and steps to follow in obtaining a glucose reading. (Consult with the operations manual which can be obtained from Boehringer Mannheim. 1-800-858-8072)

6.1.2 Venipuncture Procedure

1) Position subject in comfortable chair in an environment free from distraction.

2) Query subject about fasting state. Record time since last food or beverage on GTT check list (appendix 26). If subject is not fasting, record time and note in comment section what foods or beverage were consumed that morning. Be sure to include any additives like cream, sugar, or artificial sweeteners if a beverage was consumed. Regardless of fasting state, precede with drawing procedure.
3) Inform subject about procedure. Use left arm if possible.

4) Assemble all materials; have extra tubes within reach.

5) Apply tourniquet; have subject close fist and palpate for vein. (A vein feels like an elastic tube and bounces when pressure is applied). If the presence of vein is questionable, remove or loosen tourniquet. If the structure remains, it probably was not a vein; if it disappears assume it was a vein. Another technique to assist in locating a vein is to moisten the skin with alcohol as it will decrease the friction and may aid in the palpation of a vein. If the tourniquet has been on for 2 minutes, loosen and reapply before performing venipuncture.

6) Cleanse skin over vein thoroughly using a circular motion from center to periphery. Dry with sterile gauze.

**DO NOT TOUCH SKIN AFTER CLEANSING**

7) Put gloves on; fit needle into Vacutainer sleeve and place lavender top tube into sleeve.

8) Pull skin taut 2 inches below site to keep vein from rolling. With bevel in upright position, enter vein and then push the tube forward as far as it will go. Hold needle in the same direction as vein and at a 15 degree angle to vein.

9) After blood begins to flow, loosen tourniquet.

If blood does not begin to flow try the following:

a) Move needle slightly in or out.

b) Rotate needle slightly or lift needle to move bevel away from wall of vein.

c) Try another tube.

d) Loosen tourniquet; blood flow may be impeded if tourniquet is too tight.

* Be sure to watch for signs of hematoma from a vein. If there is any indication of hematoma, immediately remove tourniquet and needle. Place pressure and/or ice pack on site for 10 minutes. If the first attempt to obtain blood is unsuccessful with the subject’s permission try again on the opposite arm. The same technician should not attempt a venipuncture more than twice.

10) When first tube is filled, remove tube and replace with the next tube. Invert filled tubes 3x and place on ice.

11) Proceed with additional tubes in this order:

- **Fasting:**
  - 3(10ml) lavender
  - 1 (3 ml) gray

- **2 Hr:**
  - 1 (3ml) gray
  - 1 (5ml) lavender

If GTT not done add 5ml lavender top tube at fasting.
12) After drawing the last tube, remove tourniquet. Place gauze on site of needle entry and quickly withdraw needle. Apply pressure to site. Ask subject to bend arm and hold gauze pad with pressure until told to relax.

13) Record the time the fasting draw is completed on the GTT check list.

14) Serve glucose beverage; instruct subject to consume it within 3 minutes. Record time on GTT check list.

15) Confirm that bleeding has stopped and apply pressure bandage at venipuncture site. If bleeding has not stopped, elevate arm and continue to apply pressure until it stops.

16) Affix preprinted labels to tubes, making sure that ID# and tube designation are correct.

17) Give subject labeled urine specimen cup and instruct him to void into container. Inform him where to leave the container.

18) Remove gloves, wash hands, and precede to next patient.

6.2 SAMPLE PREPARATION, STORAGE AND SHIPPING

The laboratory procedures described in this manual are being implemented in the PENN MED LABORATORIES of the Medlantic Research Foundation.

6.2.1 General Rules for Handling Sample for Lipid and Other Measurements

One important precaution which should always be kept in mind in the handling of sample for lipids and lipoprotein measurements is that the blood should be kept cold (either in the refrigerator or on ice) as soon as the sample has been collected. Plasma should be separated from the cells within a few hours. Plasma samples should not be allowed to freeze and thaw during any of the handling steps.

6.2.2 Processing of Blood Samples and Urine Sample

The following flow diagram indicates exactly the blood collection procedure of the protocol. A check list is available in the Appendix 27a and should be completed for each participant. Appendix 27b is a check list for quality controls which would require a number of additional tubes of blood.
Figure 6.1  Processing of Blood Samples and Urine Sample

**Precautions:** Gloves should be worn at all times when blood and urine samples are being handled.

### FASTING

- **Three [10-ml] Lavender-top tubes**
  - Centrifuge (1500 RPM, 10min, 4°C)
  - Plasma
  - **Blood Cells**
    - Recap and ship cold on Blue Ice
    - 10 [2-ml] cryovials (approx. 0.300 ml each)
    - Store frozen and ship on Dry Ice
  - 1 [14-ml] tube (approximately 7ml)
    - Ship cold on Blue Ice

- **One [3-ml] Gray-top tube**
  - Centrifuge (1500 RPM, 10min, 4°C)
  - G0
  - 2 [2-ml] cryovials with yellow tops stored frozen
  - Fasting Glucose
  - Ship frozen on Dry Ice

### 2-Hour SAMPLE

- **One [5-ml] Lavender top tube**
  - Whole Blood
  - Hemoglobin A-Ic stored cold
  - Ship cold on Blue Ice
  - Do Not Process

- **One [3-ml] Gray-top tube**
  - Centrifuge
  - 2-hr Glucose G2
  - Two [2-ml] cryovials with red tops
  - Store frozen
  - Ship frozen on Dry Ice
6.2.3 Blood Processing

1. Label vials and tubes for each patient as follows (this should be done before beginning the blood processing)

<table>
<thead>
<tr>
<th>Container</th>
<th>Label</th>
<th>Shipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting Samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>From the 3 x [10ml] Lavender Tubes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-ml tube</td>
<td>ID</td>
<td>cold</td>
</tr>
<tr>
<td>2-ml Cryovials</td>
<td>ID</td>
<td>frozen (x10)</td>
</tr>
<tr>
<td>From the [3-ml] Gray Top Tube</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-ml cryovial with yellow top</td>
<td>ID</td>
<td>frozen (x2)</td>
</tr>
<tr>
<td>From Urine Sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-ml Tube</td>
<td>ID</td>
<td>frozen</td>
</tr>
</tbody>
</table>

2. Place vials and tubes in order each on ice

3. Centrifuge all blood tubes except the [5-ml] Lavender top tube taken at the 2-hr, at 4°C 10 minutes, 1500 RPM spin (500xg)

4. Remove from centrifuge and place on rack on ice

5. Remove caps from 3 [10-ml] lavender top tube

6. With a disposable transfer pipet, place approximately 6-7 ml into the 14ml tube to be shipped refrigerated every week. With the same pipet transfer all the remaining plasma into the 10 (2ml) cryovials remaining (approx. 0.3 ml each). Be very careful not to disturb the cell pellet in the bottom of the Lavender top tubes.

7. Do not freeze the [14-ml] test tube, place in refrigerator for shipment once a week. Make sure that the cap has been snapped tightly and not just closed to prevent leakage.
8. Recap 3 lavender tubes. Do not freeze. Place in refrigerator and ship on blue ice weekly.

9. Discard transfer pipet #1,


11. With a fresh transfer pipet #2, transfer plasma to the two cryovials for fasting glucose and creatinine with Yellow-colored caps.

12. Discard tube with red cells and transfer pipet #2.


14. With a third fresh pipet transfer plasma to two cryovials for the 2-hr glucose. These tubes will be indicated by Red-colored caps.

15. Discard tube and transfer pipet #3.

16. The [5-ml] Lavender top tube taken at the 2-hr sample should be sent refrigerated every week to the Core Lab UNPROCESSED.

17. Take patient’s urine sample, pour approximately 8 ml into tube labeled “URINE” and discard the rest

18. Two zip-lock bags will be needed for each participant.

Bag A will be used for cold shipment and should contain
   * 1 [14-ml] plasma tube
   * 1 [5-ml] Lavender top tube unprocessed
   * 3 recapped Lavender top tubes

Bag B will be used for frozen shipment and should contain
   * 10 [2-ml] cryovials for plasma
   * 2 [2-ml] cryovials with yellow-colored caps
   * 2 [2-ml] cryovials with red-colored caps
   * 1 [14-ml] urine sample

The two lab checklists in Appendix 27 a and b will be used by the staff who process the blood and urine sample. Appendix 27c illustrates the recommended flow of samples for two participants using the Workstation.

One [10-ml] QA Lavender top tube should be processed and fresh plasma transferred to 5 [2-ml] cryovials with the proper ID label. The second [10-ml] QA lavender top tube should be processed and fresh plasma transferred to a plastic screw cap test tube. The QA Gray top tube should be also processed as any regular Gray top tube and the plasma placed into two [2-ml] cryovials with red top. These 7 [2-ml] cryovials and the [14-ml] urine tube will be kept frozen in a separate zip-lock bag and sent in the next Dry-Ice shipment. The [5-ml] QA Lavender top tube taken at 2-hour and the screw cap plastic test tube containing the plasma from the second lavender top tube should be placed in a separate zip-lock bag and sent directly to the Core Laboratory with the next shipment on Blue-Ice.
6.2.4 Equipment and Supplies

1. Equipment: A refrigerated clinical centrifuge pre-cooled at 4°C will be required for the separation of plasma from the cells. The centrifuge rotor should have adapters for 13x100 tubes.

Alternatives would include the placement of a non-refrigerated centrifuge in a standard refrigerator via an extension cord.

2. Supplies:

A description of the various tubes and supplies that would be needed in the study is presented. Except for the Cryovials for frozen samples, all other items can be substituted with equivalent items from the local distributor.

<table>
<thead>
<tr>
<th>Items</th>
<th>Size</th>
<th>Packaging</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Tubes w/push-on cap</td>
<td>14-ml</td>
<td>1000/case</td>
<td>polypropylene</td>
</tr>
<tr>
<td>Cryovials w/screw cap</td>
<td>2-ml</td>
<td>500/case</td>
<td>Nalgene cryoware</td>
</tr>
<tr>
<td>Transfer Pipets</td>
<td>7-ml</td>
<td>500/box</td>
<td>polyethylene</td>
</tr>
<tr>
<td>Vaccutainer, EDTA</td>
<td>10-ml</td>
<td>1000/case</td>
<td>15% solution BD6457</td>
</tr>
<tr>
<td>Vaccutainer, EDTA</td>
<td>5-ml</td>
<td>1000/case</td>
<td>15% solution BD6507</td>
</tr>
<tr>
<td>Vaccutainer, fluoride</td>
<td>3-ml</td>
<td>1000/case</td>
<td></td>
</tr>
<tr>
<td>Vac. Multiple Sample Needles</td>
<td>21G</td>
<td>1000/case</td>
<td>BD7212</td>
</tr>
<tr>
<td>Vac. Reusable Holder</td>
<td></td>
<td></td>
<td>(10 free with each case of tube)</td>
</tr>
<tr>
<td>[2x2] Sterile Gauze</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol Wipes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Latex gloves</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tourniquet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Needle disposal device</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.2.5 Shipping

1. Shipment Schedule

   All refrigerated samples should be shipped in frozen dry ice within 5 days of draw.

   Frozen samples should be shipped on dry ice once every 2 weeks. When packing, place at least 8 - 9 lbs. dry ice in the box. Pack tightly and do not add any other packing material.

   For either fresh or frozen samples, put the shipping slip in a plastic bag.

2. Shipping Slip

   A pre-printed form will be available to each clinic for inclusion with each shipped container. Information required for each participant would include the ID code, the number of plasma tubes, the number of special blood tubes, and the number of blood cell tubes. Place check mark next to the ID number in the Frozen Shipment Form of diabetics receiving insulin. Extra labels should be mailed along with the bag containing the unfrozen plasma and the whole blood (5 ml PTT) drawn on each patient. These labels will be used for dispensing the whole blood into freezer containers for hemoglobin A1c assays. A copy of the shipping slip should be retained by the originating clinic (Appendix 27d).

   Upon receipt of the samples, a status check list will be sent back to the central office of PI's (or any pre-designated individual), presumably by FAX. Key information to watch for is the condition of the samples. If the temperature is inadequate, it would be important to increase the number of cold packs for subsequent shipment. The results for the laboratory measurements of each participant will be returned within 7 working days in the shipping package along with the cold packs to be re-used. The final results including all laboratory measurements will be available only every two months.

3. Shipment Address

   Refrigerated samples (Wet Ice) for analyses should be sent via airfreight, overnight delivery, to the following address for receipt between 9:00 AM to 5:00 PM EST, Monday through Friday:

   Penn Med Labs, Suite 150
   650 Pennsylvania Ave, S.E.
   Washington D.C. 20003
   Telephone: (202) 675-4760
   Fax: (202) 675-6042
   Attn: EDNA ROSS
Arrangements can be made for the samples to be picked on Saturdays or holidays at the Federal Express office

Federal Express  
201 Pennsylvania Ave S.E.  
Washington D.C. 20003

Frozen samples (Dry Ice) to be shipped every 2-4 weeks should be sent to:

Dr. Michael Paidi  
George Hyman Research Building  
Medlantic Research Foundation  
108 Irving Street NW  
Washington DC 20010  
Phone (202) 877-6530  
FAX (202) 877-3209

Mrs. Edna Ross is the laboratory supervisor and will be able to answer any question regarding the status of the shipment. Special shipment for weeks involving a legal holiday should be coordinated with Mrs. Ross.

6.3 LABORATORY PROCEDURES

6.3.1 Plasma Lipids

All lipid determinations will be carried using the Hitachi 705 Clinical Chemistry Analyzer using enzymatic kits obtained from Boeringer Mannheim Diagnostics.

1. CHOLESTEROL ASSAY

PRINCIPLE:

All cholesterol esters present in serum or plasma are split quantitatively (100%) into free cholesterol and fatty acids by cholesterol esterase:

\[
\text{cholesterol esterase} 
\]

\[
\text{cholesterol esters} \quad \rightarrow \quad \text{cholesterol} + \text{fatty acids} 
\]

In the presence of oxygen, free cholesterol will be oxidized by cholesterol oxidase to cholest-4-en--one:

\[
\text{cholesterol oxidase} 
\]

\[
\text{cholesterol} + O_2 \quad \rightarrow \quad \text{cholest-4-en-3-one} + H_2O_2 
\]
The hydrogen peroxide reacts in the presence of peroxidase (POD) with phenol and 4-aminophenazone to form an o-quinone imine dye:

\[
\text{POD} \\
\text{H}_2\text{O}_2 + \text{phenol} + 4\text{-aminophenazone} \rightarrow \text{o-quinone imine dye} + 2\text{H}_2\text{O}_2
\]

The intensity of the color formed is proportional to the cholesterol concentration and can be measured photometrically.

**SPECIMEN COLLECTION:**

Serum: Lipemic samples do not interfere with cholesterol recovery. Hemolysis up to 200 mg/100 mL has no effect on the assay. Bilirubin up to 12 mg/100 mL shows no interference.

Plasma: EDTA is the recommended anticoagulant. Do not use citrate, oxalate or fluoride. Cholesterol is stable in specimens for up to six days at 2-8°C or at room temperature(20-25°C).

**REAGENTS:**

The Cholesterol Reagent is intended for in vitro diagnostic use. The components of Cholesterol System Pack for HITACHI 705 include:

Cholesterol Reagent

Reactive Ingredients:

- 0.18 mmoles 4-Aminophenazone
- 0.73 mmoles 3,4-Dichlorophenol
- 1.09 mmoles Phenol
- ≥45 U Cholesterol oxidase (Nocardia erythropolis) (25°C)
- ≥72 U Cholesterol esterase(microorganism) (25°C)
- ≥36 U POD (horseradish) (25°C)

Nonreactive Ingredients: Buffer, stabilizers

**Precautions:** DANGER - TOXIC. NEVER PIPETTE BY MOUTH. In case of contact flush affected areas with copious amounts of water. Get immediate medical attention for eyes, or if ingested.

**Storage:** Store unopened at 2-8°C.

**Preparation of Working Reagent:**

1. For R1 Working Solution, reconstitute the contents of one Bottle of Cholesterol Reagent to the shoulder (180 mL) with distilled or deionized water. R1 Working Solution is stable for four weeks at 2-12°C or 7 days at (20-25°C)

2. R2 Working Solution is not required
INSTRUMENT SETTINGS:

CHANNEL SETTING: (see page 7 of Operators’ Manual)
CHANNEL NO: 1
TEST CODE: 11

CHEMISTRY PARAMETERS: (see page 6 of Operators’ Manual)

TEST CODE: 11 (CHOL)
ASSAY CODE: 1 (ENDPOINT)
SAMPLE VOLUME: 5 μL
R1 VOLUME: 500 μL - ______
R2 VOLUME: 1-1-N
R3 VOLUME: ---
WAVELENGTH 1: 600 nm
WAVELENGTH 2: 505 nm
RGT. BLK. ABS: 0
RGT. BLK. CONC: 0
STD. CONC.: * - * - <blk>
FACTOR: 0
STD. ABS. ALLOWANCE: 10%
NORMAL RANGE L: 140 mg/dL
NORMAL RANGE H: 240 mg/dL
ABS. LIMIT (RATE): 0
CONTROL ID NO.: 1 - 2 - <blk>

* Values will be based on PreciCal Normal and Abnormal as used during the daily calibration. Might be expected to change every 6 months.

CONTROLS:

On each tray position one and two will be the high and low QC pools and in the middle three more QC pools will be run.

CONTROL POOLS:
1 vial reconstituted every three days.
Add 10 ml DW using calibrated pipet.
Mix well on mixer 30 minutes.

AFTER RUN:
Transfer lab sequence numbers to tape.
Tape and list go to data entry.
Data are double entered.

2. TRIGLYCERIDE

PRINCIPLE:

Esterase hydrolyzes triglycerides to glycerol and fatty acids. The glycerol is then oxidized to dihydroxyacetone phosphate and hydrogen peroxide. In the presence of peroxidase, the peroxide reacts with 4-aminophenazone and 4-chlorophenol in a Trinder reaction to a colorimetric endpoint.
1) triglycerides + 3H₂O $\rightarrow$ glycerol + fatty acids

GK

2) glycerol + ATP $\rightarrow$ glycerol-3-phosphate + ADP

GPO

3) glycerol-3-phosphate + O₂ $\rightarrow$ dihydroxyacetone phosphate + H₂O₂

peroxidase

4) H₂O₂ + 4-aminophenazole + 4-chlorophenol $\rightarrow$ 4-(p-benzoquinone-

monoimino) phenazone + 2H₂O + HCl

SPECIMEN COLLECTION:

Plasma: EDTA will be used as anticoagulant.
Triglycerides are stable in plasma for 3 days at 2-8°C or frozen at -20°C.

REAGENTS:
The Triglycerides (GPO) Reagents are intended for in vitro diagnostic use.
The components of the Triglycerides (GPO) System Pack for HITACHI-705 include:

1. Buffer/Enzymes (enzymes added on reconstitution)

Reactive Ingredient:
0.35 mmol 4-chlorophenol

Nonreactive Ingredients:
Buffer, preservative, detergent

Precautions:
WARNING - TOXIC. AVOID INHALATION AND CONTACT WITH SKIN OR MUCOUS MEMBRANES. In case of contact, flush affected areas immediately with copious amounts of water. Contains sodium azide; if drain becomes clogged, clean with 10% sodium hydroxide.

Storage: Store tightly closed at 2-8°C.

la GPO

Reactive Ingredients:
≥0.05 mmol ATP
0.035 mmol 4-Aminophenazole
≥300 U Esterase (E.C. 3.1.1.13; microorganism)
≥250 U Glycerol phosphate oxidase
≥20 U Glycerokinase (EC 2.7.1.30; microorganism)
≥15 U Peroxidase (E.C. 1.11.1.7; horseradish)
Precautions: Exercise the normal precautions required for the handling of all laboratory reagents.

Storage: Store tightly closed at 2-8°C.

Preparation of Working Reagents:

1. For R1 Working Solution, connect one Bottle 1 (Buffer/Enzymes) to one bottle 1a (GPO) using one of the enclosed adapters. Mix by gentle inversion. Completely dissolve the lyophilizate in the buffer. R1 Working Solution is stable for 2 weeks at 1-12°C or 2 days at room temperature (20-25°C).

2. R2 reagent is not required.

INSTRUMENT SETTINGS:

**CHANNEL SETTING** (see page 7 of Operators’ Manual)

| CHANNEL NO: | 2 |
| TEST CODE:   | 33 |
| TEMPERATURE: | 37°C |

**CHEMISTRY PARAMETERS** (see page 6 of Operators’ Manual)

| TEST CODE:     | 33 (TRIG) |
| ASSAY CODE:    | 1 (ENDPOINT) |
| SAMPLE VOLUME: | 5 |
| R1 VOLUME:     | 500 -N |
| R2 VOLUME:     | 1-1-N |
| R3 VOLUME:     | --- |
| WAVELENGTH 1:  | 700 |
| WAVELENGTH 2:  | 505 |
| RGT. BLK. ABS: | --- |
| RGT. BLK. CONC:| 0 |
| STD. CONC.:    | * - * - <blk> |
| FACTOR:        | --- |
| STD. ABS. ALLOWANCE: | 10% |
| NORMAL RANGE L: | 40 mg/dL |
| NORMAL RANGE H: | 150 mg/dL |
| ABS. LIMIT (RATE): | 0 |
| CONTROL ID NO.: | 1 - 2 - <blk> |

* Values will be based on PreciCal Normal and Abnormal as used during the daily calibration. Might be expected to change every 6 months.

<blk> blank, no entry

If specimen exceeds 500, dilute sample with physiological saline, and re-run.

**CONTROLS** low, medium and high. Standardized controls are run with each assay.

**NUMBERING & DATA**: Same as for Cholesterol, page 9.
3. FREE GLYCEROL

PRINCIPLE:

This procedure will measure only the free glycerol. By subtracting the free glycerol from total, triglyceride glycerol is computed. During the assay glycerol is reacted with ATP in the presence of GK to form glycerol-3-phosphate plus ADP.

\[
\text{GK} \\
\text{Glycerol} + \text{ATP} \quad \text{---------} \quad \text{glycerol-3-phosphate} + \text{ADP}.
\]

The ADP reacts with phosphoenolpyruvate (PEP) in the presence of pyruvate kinase (PK) to form ATP and pyruvate.

\[
\text{PK} \\
\text{ADP} + \text{PEP} \quad \text{---------} \quad \text{ATP} + \text{pyruvate}
\]

Pyruvate reacts with nicotinamide-adenine-dinucleotide-reduced (NADH) in the presence of lactate dehydrogenase (LDH). The amount of NADH oxidized during the reaction is equivalent to the amount of glycerol in the specimen.

\[
\text{LDH} \\
\text{pyruvate} + \text{NADH} + \text{H}^+ \quad \text{---------} \quad \text{Lactate} + \text{NAD}^+
\]

The wavelengths used are 340 nm and 660 nm.

SPECIMEN

A duplicate aliquot is taken of all specimens used for total triglyceride assay.

Reagents

1. Free Glycerol (Triglyceride Blank) - Use BMD Autoflo Triglyceride Kit, Catalog #166448. Triglyceride Kit stored at 2-8°C when not in use. Refer to kit for expiration date.

2. Extran MA 03 BMD, Catalog #7550 (Reaction cell Bath Detergent) - Use Extran MA 03 as is, no preparation required.

3. Dilute Extran - Using 0.5 ml of Extran Diluted with 200 ml distilled deionized H\textsubscript{2}O. Solution stable for 2 months at room temperature.

4. 0.1 M \text{NaOH} - Dissolve 4.0 grams of \text{NaOH} in 90 ml of distilled, deionized water. Adjust final volume to 100 ml. Store in plastic container at room temperature. Stable for 1 year.

Evaluation Parameters for Free Glycerol on Hitachi 705:

Kit Utilized:

Autoflo Triglyceride, Catalog #166448.
Reagent Preparation:

R1 Reconstitute Bottle 2 (Coenzyme Reagent) as directed in the Autoflo package insert. (15 ml distilled H₂O).

Combine 20 ml from Bottle 1 (Buffer) with 7.5 ml of Bottle 2 (15 ml Coenzyme Reagent). Use calibrated pipette. Dilute to 175 ml with distilled or deionized H₂O. Stable for 2 days at 2-8°C.

R2 Dilute 3.0 ml of Bottle 4 (Glycerokinase Reagent) to 75 ml with distilled H₂O. Store in refrigerated reagent compartment of Hitachi 705 or refrigerator at 2-8°C.

Stable: 1 day at 2-8°C.
Filter Before Use!!

Hitachi 705 Parameters:

<table>
<thead>
<tr>
<th>ASSAY CODE</th>
<th>END POINT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMPLE VOLUME</td>
<td>10 µl</td>
</tr>
<tr>
<td>R1 VOLUME</td>
<td>350 µl</td>
</tr>
<tr>
<td>R2 VOLUME</td>
<td>150 µl</td>
</tr>
<tr>
<td>WAVELENGTH 1</td>
<td>660 nm</td>
</tr>
<tr>
<td>WAVELENGTH 2</td>
<td>340 nm</td>
</tr>
</tbody>
</table>

CRT PAGE 7

CHANNEL NUMBER:6
TEST CODE: 36 - 36

Calibrator:

Precimat Glycerol, Catalog #166588, Set Point 21.0 mg glycerol/dl.
TRIG Set Point = 200.0 mg TG/dl.

Controls and procedures are identical to cholesterol assay.

4. ROUTINE START UP PROCEDURE FOR CHOLESTEROL/TRIGLYCERIDE ANALYZER

OPERATING INSTRUCTIONS:

Check water supply, reagents and temperature. Record temperature in temperature log book.

Turn power on for Hitachi, making sure that the cassette tape is in place.

Check reagents and make up all that are not sufficient for the day’s plans mix for 15-20 minutes, record lot # and expiration date in log book.
Calibration and controls must be done before performing patient samples. (see page 16)

Perform daily maintenance as listed on the log sheet. Date and sign name of person performing task, using the special cassette tape for routing maintenance. When preparing reagents, record Lot # and expiration date in reagent log book.

STANDARD WET CHEMISTRY:

1. Call up CRT Page 2, "TEST SELECTION". The cursor appears next to "Sample NO."

2. Depress (−) 1, then Enter.

3. The line now displays "Blank".

4. Depress TEST SELECTION KEYS for chemistry tests for which blanks are to be performed, then ENTER.

5. The line now displays "STD". (See page 7-27 of Operators' Manual for more details).


7. The line displays 1-001, depress the down arrow (↓) key to move the cursor to the clear "LINE".

8. Depress YES, then depress ENTER.

NOTE: This insures that any patient test request from the previous run are deleted from memory.

9. Call up CRT, Page 1, "START AND MONITOR".

10. Type DATE, then ENTER.

11. Ensure that the number 1 is displayed next to the "SAMPLE NO." line. If the number 1 is not there, depress number 1.

12. Using down arrow (↓) key - cursor displays "CALIBRATION LINE".

13. Depress YES, then ENTER. This instructs the system to perform the calibration sequence on the selected tests.

14. "MULTI-STD" line, depress YES, then ENTER.

15. "SERUM INDEXES", depress NO.

16. Use down arrow (↓) key to "CONTROL INTERVAL", depress numeric key corresponding to the desired interval for processing patient samples between control samples, the depress ENTER.
17. Verify that INCUBATION temperature is 37°C.

18. Fill a sample cup with physiological saline and place it in position B in the inner ring of the sample tray.

19. Fill a sample cup with multi-constituent standard (calibrator) and place it in the designated position (S1, S2 or S3) in the inner ring of the sample tray.

20. Place controls in position C1, C2 or 3.

21. Depress the **START** key.

22. After about three (3) minutes, the first result will be printed. After completion of run, record in Q.C. book.

**Calibration of Hitachi:**

Use Precical (BMD) for C & TG.
For HDL-C use 50 mg/dl standard (BMD).
For glycerol use 200 mg lab standard (BMD).
Saline blank.
Run level 1 and 2 controls, plus precical level I normal and abnormal for each analyte.
Request calibration procedure.
Record in log.
Recalibrate every 4-6 hours.

5. **HDL-Cholesterol**

**PRINCIPLE:**

In the presence of MnCl₂ and heparin, Chylomicron, VLDL and LDL are selectively precipitated, leaving only HDL in solution. The precipitated lipoprotein are sedimented by centrifugation and the clear, HDL-containing supernatant is recovered for measurements of cholesterol.

**HDL-CHOLESTEROL STANDARDS:**

Preciset Cholesterol standards containing 50 and 150 mg/dl are obtained from Boehringer Mannheim Diagnostics. Using the 150 mg/dl standard, two new standard solutions are obtained to reflect the lower range required for the determination of plasma HDL.

<table>
<thead>
<tr>
<th>Final conc.</th>
<th>Stand.</th>
<th>Final volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mg/dl</td>
<td>4.16ml</td>
<td>25ml</td>
</tr>
<tr>
<td>75 mg/dl</td>
<td>12.5ml</td>
<td>25ml</td>
</tr>
</tbody>
</table>

The standard solutions should be kept at 4°C.

Three extra pools with known HDL values are placed at the beginning, middle and end of each HDL rack.
PRECIPI TATION PROTOCOL:

a) Heparin-Manganese precipitation should be performed as soon as possible after sample collection, preferably on the day the samples are drawn. If necessary, however, plasma may be stored at 4°C for no longer than 7 days before the HDL-fraction is prepared. In most cases, storage related changes are detectable, but small during this time.

b) Manganese Chloride (MnCl₂·4H₂O, Sigma M-3624) should be stored in a vacuum desiccator to minimize water uptake. Dissolve 10.0127 gm of manganese chloride in 50 ml with fresh deionized water.

c) Working reagent should be made fresh (every 2 days) using 0.6 ml of Sodium Heparin (20,000 usp units/ml LyphoMed Cat.No. 9155-01) and 5 ml of the MnCl₂ solution.

d) HDL Blank is obtained by adding 200 mg of EDTA to 100ml of normal saline solution which is subsequently titrated to pH 7.0 with 1N NaOH.

e) Transfer 500ml of HDL blank or unknown to individually labeled microfuge tube (1.5ml capacity). Add 50ul of heparin/manganese reagent (see above). Vortex vigorously and allow samples to stand at 4°C for overnight. Sediment the precipitate by centrifugation at 7,500 x g for 30 minutes (4°C). The supernate can be decanted into another properly labeled tube. The HDL supernate is ready for HDL-cholesterol determination or can be stored at 4°C.

Calibration:

a) Reconstitute A-GENT Cholesterol using the volume of distilled water given on the vial. Add 210ul of EDTA (150mg/ml). Mix gently to avoid bubbles or foaming. Pure cholesterol standards (25, 50 and 75 mg/dl) are needed to establish the standard curve.

b) Place 500ul of HDL blank into the 01 cup and 500ul standards, control sera and unknowns into subsequent cups. DO NOT USE SERA-SEAL ON STANDARDS AS IT WILL DILUTE THE CONCENTRATION.

c) Determine the CF as for Cholesterol determination in the previous section.

d) Proceed exactly as for Cholesterol determination.

6. LDL-Cholesterol

PRINCIPLE:

The VLDL fraction is isolated by ultracentrifugation at density (d < 1.006 gm/ml). The cholesterol in the bottom fraction is measured and the calculation of LDL-CHOL is:
LDL-CHOL = CHOL(d > 1.006) - (HDL-CHOL)

ULTRACENTRIFUGAL ISOLATION OF PLASMA VLDL

Specimen: The specimen should be freshly drawn plasma (or serum) which has not been frozen. If specimens must be shipped to the laboratory, they should be packed unfrozen on water ice. Ultracentrifugation should be carried out as soon as possible, but samples may be stored at 4°C for up to 7 days if necessary.

Set-up for VLDL Spin: Use the Beckman 40.3 or 50.3Ti rotor with pollyallomer tubes (2.5” x 0.5”, capacity 6ml).

* Pre-cool the rotor to 10°C
* Label ultracentrifuge tubes and place them in a test tube rack in an ice bath.
* Pipet 5ml of plasma into the appropriately labeled ultracentrifuge tube (A smaller volume of plasma may be used if insufficient sample is available - the actual volume used must be recorded and any dilution factor taken into account in the final calculation).
* Carefully layer sodium chloride solution (NaCl, 0.15mol/l) over the specimen to the top of the ultracentrifuge tube.
* Seal the tube tightly. Gently squeeze the sealed tube to check for leaks.
* Place the tubes in the rotor sockets in such a way that the rotor is evenly balanced.
* Place the rotor in the ultracentrifuge and centrifuge for 18 hours at 15°C at 105,000 x g (40,000 rpm)

Recovery of the Lipoprotein Fractions:

The centrifuge must be stopped without the use of brake in order to minimize re-mixing of the fractions. The samples must be removed as soon as possible after the rotor has come to a complete stop.

* Label the 3-ml and 5-ml volumetric flasks with the corresponding sample ID number.
* Gently remove the rotor and transfer it to the area where the tubes are sliced. The rotor should be maintained in a horizontal position at all times.
* Using the extraction tool, remove each tube slowly avoiding any abrupt movement which would disturb the lipoprotein layers.
* Insert the tube into the tube slicer until the bottom edge of the tube is approximately 2.2 cm from the bottom of the tube slicer and the small dent in the tube faces away from the point of the slicer blade. Slice the tube with a quick, smooth thrust of the sharp blade. Clip off the top of the quick seal tube.

* Withdraw the top fraction through the fill-hole using a disposable pipet. Remove the top portion of the tube including the cap from the tube slicer. Transfer the remainder of the top fraction to a 3ml volumetric flask. Rinse the slicer and cap with NaCl solution. Aliquot of this fraction is used to determine CHOL and TG by the usual method allowing for the calculation of the ration VLDL-CHOL/VLDL-TG. Quantitative recovery of lipoprotein in the top fractions is difficult.

* To recover the bottom fraction, withdraw the blade slightly and remove the bottom fraction, using disposable pipet. Transfer it to an appropriately labeled 5-ml volumetric flask.

* Remove the blade completely and remove the bottom portion of the tube from the slicer. Loosen all material from the wall of the tube with the pipet tip and transfer the remainder of the bottom fraction to the 5-ml volumetric flask.

* Rinse the bottom portion of the tube with a small amount of the NaCl solution and add the washing to the 5-ml volumetric flask. Bring the contents of the flask to 5-ml with the NaCl solution. Total cholesterol in the bottom fraction is determined and used with the independently determined value for HDL-CHOL to calculate the LDL-CHOL.

7. Quality Controls

A. OVERVIEW

Penn Med Labs which is serving as the Core Laboratory for the CARDIA Study is presently standardized by the CDC for lipid measurements which include total cholesterol and triglyceride, and HDL-cholesterol. Quality Control plasma pools have been prepared and standardized and are measured. A precision check (i.e., same sample in each tray 20 times) is run each day.

Cholesterol and HDL Cholesterol

Daily standardization is accomplished using levels of reconstituted plasma, PreciCal and PreciNorm from BMD. Frozen plasma pools at three ranges of cholesterol are also used. Accuracy and precision of the cholesterol measurements are ensured by participation in several programs including the Lipid Standardization Program administered by the Centers of Disease Control in Atlanta, GA and the College of American Pathologists.
Triglycerides

The same daily standardization procedure is used for triglyceride and for cholesterol.

We follow Westgard’s rules for the evaluation of runs. In addition, we have instituted a more stringent procedure. If either level for level 2 are >SD, the tray should be rejected and repeated. If one of the three additional controls is 2SD, repeat five samples. If not with 3%, repeat whole tray. If two or more are >2SD, check pickup, do a precision check (i.e., same samples 20 times) and then repeat the tray. Before repeat, try to identify the problem with the supervisor if both runs are outside the acceptable range.

B. ACTION PLAN FOR OUT OF LIMIT CONTROLS

All analytical procedures should be reviewed for out of limit controls. Review Westgard’s Rules for acceptance or rejection. These limits are posted in the QC logs for easy reference. If an out of limit control requiring immediate action is observed, the following plan is to be employed before reporting any patient result.

1. Check all reagents for expiration, improper storage, etc. Check all instrumentation for obvious problems like insufficient gas pressure, incorrect operating temperatures, defective pipetting, lamp out, etc. Take any corrective measures necessary. Record all steps taken in the maintenance logs or QC log.

2. Repeat the assay

   a. If the QC is within limits, report the patient results.
   b. If the QC is still unacceptable, notify the laboratory supervisor. No patient results may be reported in this situation. The supervisor should make sure the assay is performing correctly before any additional patients are analyzed.

3. It is essential that any remedial action taken be documented. Out of limit controls should be circled and initialed in the QC log book. The specific steps taken should be recorded either below or on the reverse page. For example, if a single control is between 2 and 3 SD observe the next run, that should be noted in the logbook. If an assay is repeated, write it down. It must be documented that patient results were not reported for any out of control assay.

4. Note: For cholesterol and triglyceride assays, tray should be rejected if control pools are not within +/- 1% of the reference value.

C. WESTGARD’S MULTI RULES FOR QUALITY CONTROL

1:3s Reject when one observation exceeds the mean by +/- three or more standard deviations (SD).
2:2s  Reject when 2 consecutive observations exceed the mean by plus or minus two or more standard deviations.

R:4s  Reject when one control observation in the run exceeds its mean by 2 SD and the next control exceeds its mean by minus 2 SD. (A difference of 4 SD).

10:mean  Reject when ten consecutive control observations fall on one side of the mean.

Means and standard deviations for each control pool are determined after at least 10-15 separate days of assay. For each control pool for each assay, a graph is constructed showing the mean and the two standard deviation +/- limits. Values for each days’ control pool are plotted on this graph as a visual means of inspecting for trends within each assay. When the supply of any one control pool is reduced to approximately 3-months’ supply, another control pool is constructed and the man and standard deviation computed so that there can be a replacement without a gap.

Please follow these guidelines for accepting control values. If control fall out of this range do not accept run and bring to the attention of the supervisor on duty.

D. RECORDING OF ACTIONS TAKEN ON OUT OF LIMIT CONTROLS

1. Refer to VI.B. on ”Action plan for out of limit controls”.

2. Record all QC results, even if it is obviously out of limit.

3. DECISION MAKING TIME:

A. If 2 out of 3 controls are within limits (+2 SD) and the third was within limits in the previous run and it is within 3 SD, accept the run. Write ”2 out of 3 in, accept run” on the QC sheet. This applies especially to Lipid assays.

B. If a single control pool is employed, and the control is out, repeat the control. If the control is still out, assay the other two controls. If the other two are in the acceptable range, follow A above, recording this decision in the QC log.

C. If all controls are out, refer to our action plan for out of limit controls. Document which corrective actions were taken; i.e., recalibration, faulty probe, etc. on the QC sheet. Repeat your QC and also record these acceptable results in the QC log.

D. If an assay is rejected and no results are reported because the assay will be repeated on another day, write ”assay rejected, no results reported”.
6.3.2 Apolipoproteins

1. Sandwich ELISA for apoB

Pooled plasma was used to isolate LDL by ultracentrifugation and will be coupled to CNBr-activated Sepharose 4B for the isolation of specific IgG against apoB. The best fraction is conjugated with alkaline phosphatase and the IgG fraction of weaker specificity is used to coat the plates.

Step 1 (Friday) Coat the Plates

Take out frozen vial containing specific IgG against apoB from the -80°C freezer. Allow to thaw at room temperature. DO NOT RE-FREEZE IgG. Prepare a 1:500 dilution of the IgG using coating buffer. You will need 10 ml of dilute IgG per plate or 50 ul of IgG. Add 100 uL of anti-apoB IgG (1:500 in coating buffer) into 96 wells Immunolon II flat bottom plate. Cover and store it in a humidifier at 4°C.

Step 2 (Monday) Block the Plates

Wash the plate 3 to 4 times with wash buffer with alternate drying in between wash by placing it upside down on a couple layers of paper towel. Add 200 uL of Blocking Solution and cover and store it in a humidifier as in step 1. Set up a 1/100 dilution of standards, control plasma samples, and unknown samples in Wash Buffer and store them at 4°C. Dilute sample. Recommended dilution of plasma for apoB is 1:30 K.

Step 3 (Tuesday) Sample Application

Wash the plate 3 to 4 times and dry it as in step 2. Add 100 uL of standard, plasma or unknown to the appropriate wells. Cover and store it as in the previous step.

Step 4 (Wednesday) Application of Enzyme-linked Antibody

Wash the plate 3 to 4 times and dry it. Prepare a 1:500 dilution of the enzyme-linked IgG (stored in the refrigerator). You will need 10 ml of the diluted preparation for each plate. Add 100 uL of the diluted enzyme-linked IgG preparation to each well.

Step 5 (Thursday) Application of Substrate

Wash the plate 3 to 4 times and dry it. Prepare substrate buffer at a concentration of 1 mg/ml. You will need 10 ml of substrate buffer for each plate. Substrate (Sigma P104-105) is available as 5 mg per pellet and should be kept in dark bottle in the desiccator. Once the substrate is added put the plate in the humidifier at room temperature and let the plate develop. The development process should be carried out away from light.
Step 6 (Thursday or Friday)  

Reading the Plates

Once the highest concentration of the standards reaches an O.D. of 1.0, the plate should be read. It should take approximately 3 hrs for the color to develop. Slow color development might occur if the room temperature becomes too low, in which instance the plate can be placed in the refrigerator and read the next morning. If this becomes a consistent problem it would indicate that one of the IgG preparations might have been denatured. A new batch should be used.

Step 7 (Friday)  

Data Analysis

Plot OD[405nm] vs the concentrations of the apolipoprotein standards (in ng/mL). Extrapolate the concentration of the apolipoprotein in the unknown sample from the standard curve. STATGRAPHICS will be used as a PC-based package for data analysis.

2. Sandwich ELISA for apoA-I

The same 7 steps will be used for the apoA-I ELISA with the appropriate antibody. Pooled human HDL isolated by ultracentrifugation was coupled to CNBr-activated Sepharose 4B for the isolation of specific IgG against apoA-I. Dilution of plasma samples is: 1 : 31 K.

3. Buffer Preparation

The following buffers will be shared by the ELISA systems. The reagents and supplies required for the immunoassay are listed in B.2.5

a) Coating Buffer (Tween or milk powder) 50 mM PO₄₋₋/0.1M NaCl/0.01% NaN₃/0.001 M EDTA pH 7.4

To prepare 6L:
Mix 498 ml 0.5 M Stock Phosphate Dibasic
101.4 ml 0.5 M Stock Phosphate Monobasic
35.04 grams of NaCl

Bring up to 5900 ml with distilled water. The pH should be around 7.2-7.4.

Add 0.6 g NaN₃ and 2.22 g EDTA.
Raise total volume to 6 liters
Mix well and store in the cold room.

b) Stock Blocking Buffer
Milk Powder Concentrate: (30 μg/mL stock)
Add 3 g milk powder/100 ml Coating Buffer, stir overnight and store it at 4°C.
c) Stock Detergent Buffer (10% Tween-20)
Add 10 ml Tween-20 concentrate to 90 ml Coating Buffer, mix it and store at 4°C.

d) Block Buffer
(1 mg/ml milk powder and 0.05% Tween)
Add 100 ml milk powder concentrate into 2800 ml Coating Buffer followed by 15 ml of the 10% Tween solution and bring up to 3 liters. Stir overnight and store it in the cold room.

e) Wash Buffer Solution:(0.1 mG/mL milk powder and 0.05% Tween)
To Make 6 liters:
Add 600 ml Block Buffer to 5300 ml Coating Buffer
Add 30 ml of the 10% TWEEN solution.
Bring up to 6 liters.
Stir and store it in the cold room.

f) Substrate Buffer Solution:
Add 242.5 ml Diethanolamine to 2 liters of distilled water followed by 0.5 g of NaN₃ and 250 mg of MgCl₂. Adjust pH to 9.8 with 1 N HCl and bring up to 2500 ml. Store in a dark bottle and keep it in the dark.

4. ApoE isoform from plasma
Plasma sample is stored at –70°C

1. Dialysis
Sample (20μl) is dialyzed at room temperature overnight
dialysis buffer

20.9g monobasic sodium phosphate
16.55g dibasic sodium phosphate
6.25g EDTA, tetrasodium salt, pH 6.8

↓
q.s. to 4l with deionized water
diaalyzed samples diluted in dialysis buffer containing 3 M urea
2. Preparation of Gel

29.1% monomer solution
   29.1g polyacrylamide → q.s. to 100 ml

0.9% dimer solution
   0.9g bis acrylamide → q.s. to 100 ml

5 ml of 29.1% monomer solution--
5 ml of 0.9% dimer solution
5.4g Urea
0.45 ml Pharmalyte pH 4.5-5.4
0.9 ml Pharmalyte pH 5-8

add 175μl 10% Ammonium Persulfate
21μl TEMED

3. Electrofocusing

Plasma samples are applied 0.5cm from the cathode

catholyte → 1M NaOH
anolyte → 1M H₃PO₄

catholyte and anolyte wicks are saturated with each solution.

LKB Ultrophor electrofocusing unit is connected with a cooling
circulation and power supply at 101V constant power

↓

after 1 hr’s running, sample application pieces are removed and the
cathode electrode wick is blotted.

* total isoelectric focusing time is 3 hrs

4. Transfer

Transfer buffer = TBS buffer
gel is covered with prewretted nitrocellulose sheet and left at room
temperature overnight
5. Immunoblotting

Nitrocellulose is removed and rinsed in TBS Buffer

\[
\text{TBS Buffer:} \quad 0.25M \text{ Nacl} \\
0.03M \text{ Tris-Hcl} \\
pH 8.0
\]

\[
\downarrow
\]

60 min incubation in 5% nonfat dry milk

\[
\downarrow
\]

90 min exposure in anti-apoE antiserum diluted 1:750 in TBS Buffer

\[
\downarrow
\]

10 min \times 3 \text{ times in TBS Buffer}

\[
\downarrow
\]

90 min incubation in anti-IgG conjugated with enzyme alkaline phosphatase diluted 1:5000 in TBS Buffer

\[
\downarrow
\]

washed in TBS Buffer three times

\[
\downarrow
\]

stained in 25mg \beta\text{-naphthyl phosphate} \\
25mg \text{ Fast Blue BB salt} \\
60mg magnesium sulfate in 50\mu l stock buffer \\
(1.8g NaOH, 3.7g boric acid/l)

Notes:

Stocking Buffer → should be stored at 4°C until use

\[
\downarrow
\]

q.s. to 4l before use (not reusable)

Dialysis is carried out overnight at room temperature

\[
\downarrow
\]

samples are diluted to 150\mu l of dialyzing buffer containing 3 Murea
When samples have precipitate → spin down and use supernatent
Apply 25μl of samples (maximal numbers of samples → 30 ~ 40 lanes)
↓
rest can be stored and frozen. Prefocusing is necessary for 15 min.

5. Quality Controls for ELISA

For the apolipoprotein assays, four replicates at one dilution are used for each plasma sample. A distribution of fractional standard deviations (FSD) is routinely obtained for every 200 samples. The 90th percentile of FSD is determined and any sample which is found to have a FSD greater than the 90th percentile will be re-measured in the next assay. Unacceptable FSD for both control samples in each plate requires all of the samples on the plate to be re-measured. All of the samples on the plates are also repeated in the next assay if the mean value of the control is outside the acceptable range (mean ± 1.60 SD).

No standardization program for plasma apolipoprotein is available at this time. The Core Laboratory is participating in a program recently initiated by the IFCC Apolipoprotein Committee and CDC Apolipoprotein Working Group. Final decision on the standardization program is not expected for several months.

In the interim, controls in the form of lyophilized plasma are obtained from the Northwest Lipid Research Center to serve as standards for the immunoassays. Controls with known concentration of apoA-I and apoB are also available from BioRad Chemicals. Frozen plasma controls are also available in the Laboratory of Lipoprotein Physiology of the Medlantic Research Foundation.

6.3.3 Glucose

PRINCIPLE:
Hexokinase catalyzes the phosphorylation of glucose by ATP:

\[
\text{HK} \\
\text{D-glucose + ATP} \rightarrow \text{G-6-P} + \text{ADP}
\]

G-6-P is oxidized to 6-phosphogluconate in the presence of NAD by the enzyme glucose-6-phosphate dehydrogenase. No other carbohydrate is oxidized:
G-6-PDH

G-6-P + NAD  --------->  gluconate-6-P + NADH + H⁺

The amount of NADH formed during the reaction is equivalent to the amount of D-glucose in the specimen and can be measured photometrically by the increase in absorbance.

SPECIMEN COLLECTION:

Plasma: Blood must be drawn in tubes containing fluoride to inhibit degradation of glucose

Glucose in plasma with fluoride is stable up to 3 days at 4°C or for up to 8 hours at 25°C.

REAGENTS:

The GLUCOSE-HK Reagents are intended for in vitro diagnostic use.

The components of the Glucose-HK System Pack for HITACHI 705 includes:

Non reactive ingredients:

27.7 mmoles Sodium chloride

Reactive Ingredients:

| 1.2 | mmoles | Magnesium |
| 433.9 | umoles | NAD |
| 365.8 | umoles | ATP |
| >1130 | U | Hexokinase (yeast) (E.C.2.7.1.1) |
| >951 | U | G-6-PDH (leuconos.) (E.C.1.1.1.49) |

CALCULATION:

The HITACHI 705 microcomputer uses absorbance measurements to calculate glucose concentration as follows:

Cx = K (Ax - Ab) + Cb

Where:

Cx = Concentration of sample
K = Concentration factor
Ax = Mean of absorbance of Sample + both reagents read at positions 30 and 31 minus mean of absorbance of Sample + the first reagent only read at positions 14 and 15**
Ab = Mean of absorbance of Blank + both reagents read at positions 30 and 31 minus mean of absorbance of Blank + First Reagents read at positions 14 and 15**

Cb = Concentration of Reagent Blank

** Corrected for reagent/sample volume by K = (sample volume + R1)/(sample volume + R1 + R2).

Linearity: Higher glucose concentrations should be reassayed after dilution with physiological saline.

Quality Control: Precitrol - N; Precitrol - Abn;
Precical - (calibrator)

Procedure for the quality control are the same as these used for cholesterol and triglyceride.

6.3.4 Insulin

PRINCIPLE:

Insulin in serum or plasma is measured by radioimmunoassay. 125-I insulin and non labeled insulin from either standards or unknown sera compete for sites on the antibody during incubation. The bound antibody-antigen complex is then precipitated through the use of a carrier (normal guinea pig serum in PEG buffer) and an antibody to the carrier (goat anti-guinea pig serum). After centrifugation, the resulting pellet is counted in a gamma counter. Quantitation is achieved by interpolation from a standard curve.

METHODOLOGY:

Total insulin in serum or plasma is measured by radioimmunoassay.

SAMPLE REQUIREMENTS:

500 ul of serum or heparinized or EDTA plasma is required. Care must be taken when using heparin as an anticoagulant since an excess will provide falsely high values (Thorell, J.I., Scand. J. Clin. Lab. Invest. 31:187, 1973). Use no more than 10 IU heparin per ml of blood collected.
CONTROLS:

Ten insulin reference controls are used, three each from Bio-Rad and Diagnostics Products Corp. (DPC) and 4 plasma pools. Insulin concentrations should be in the low, medium and high range, i.e., approximately at the calculated ED$_{80}$, ED$_{50}$, and ED$_{20}$ levels of the standard curve. These reference plasmas must be run in triplicate with each assay.

Each month the mean and standard deviation (SD) for QCs are calculated for the last 10 assays or for the month, whichever provides the greater number of assays for evaluation. The %CV and Range (+- 2 SDs) are calculated and used to evaluate all new assays for the coming month. If three of the QCs fall outside of +- 2 SD, data can not be reported. If any QCs remain outside of limits on repeat testing, hydrate new vial and see supervisor. Upon reconstitution with distilled water, the QC should be stored at -20°C in small aliquots.

QC 1, 2, 3:  
BioRad, (714) 630-6400100  
Alfred Nobel Dr.  
Hercules, CA 94547  
Cat.# C370-5, $139.00/3 vials  
Reconstitute the reagents with 5 ml of water

QC 4, 5, 6:  
Diagnostics Products Co. (DPC)  
1-800-654-3707  
5700 West 96th St.,  
Los Angeles, CA 90045  
Cat.# CON6, $40.00/6 vials  
Reconstitute the reagents with 6 ml of water.

REAGENTS:

A. Buffer 0.05M Phosphate buffered saline, pH 7.4

a. NaCl - 9g/liter  
b. Na$_2$HPO$_4$ - 7H$_2$O - 12.35g/liter or Na$_2$HPO$_4$ (anhydrous) 6.54 g  
c. KH$_2$PO$_4$ - 0.65g/liter Sigma P0662 Potassium Phosphate  
   Monobasic $16.00/500g  
d. EDTA - 9.3 g/liter  
e. EMSTA - 0.1g/liter Kodak 135 8456 $59.00/25g, Ethyl  
   Mercurithiosalicylic Acid, Sodium Salt  

EDTA is not dissolved easily. When pH is adjusted to 7.4  
with 5N or 10N NaOH, EDTA can be dissolved quickly.

a1. Assay Buffer, 0.05M Phosphosaline, pH 7.4, 1% BSA. Use only  
   Sigma R1A grade BSA:
Sigma A7888, Albumin, Bovine, RIA grade, Fraction V Powder $84.15/50g, $140.45/100 g. Stable 1 month. Store refrigerated.

a2. Buffer for the second antibody and the carrier (NGPS), 0.05M Phosphosaline, pH 7.4, 3% PEG 8,000
PEG 8,000: Sigma P2139, Polyethylene Glycol 8,000, $10.35/500g Store at 4°C.

a3. Wash Buffer to wash the precipitate, 0.05M Phosphate buffered saline, pH 7.4, without BSA, without PEG.

B. First Antibody - Guinea Pig Anti Porcine Insulin Serum. Dilute to obtain a 34-45% B0. Binding of total label insulin. Store at -20°C. Obtained from:

Linco Research, Inc. (314) 527-2188
P.O. Box 641, Eureka, Missouri 63025
$60.00/1000 tubes.

For lot 122-845-P, reconstitute the content of the vial with 1 ml of assay buffer. Aliquot to small vials and store at -20°C. For use in assay, further dilute to 1:100 with assay buffer and add 100ul per tube.

C. Human Insulin Standards -

a. Obtained from Dr. Ronald E. Chance of Eli Lilly and Co. (317) 276-4233. See the Appendix for details. This is provided as 0.57 U per vial with 1 mg of Human Serum Albumin as a carrier. Add 5.7 ml of 0.01 N HCl to produce a 100 mU/ml stock solution. Store at 4°C., DO NOT FREEZE. Insulin for treatment of human diabetes cannot be used. It may not be accurately quantitated.

b. Preparation of Working Standards.

Stock A: Add 100 ul of Stock Insulin (100 mU/ml) to 39.9 ml of assay buffer to obtain the working stock at a concentration of 250 uU/ml.

Dilution Table

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration uU/ml</th>
<th>Stock A ml</th>
<th>Buffer ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>200</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>02</td>
<td>100</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>03</td>
<td>50</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>04</td>
<td>25</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>05</td>
<td>12.5</td>
<td>0.5</td>
<td>9.5</td>
</tr>
<tr>
<td>06</td>
<td>6.3</td>
<td>0.2</td>
<td>9.8</td>
</tr>
<tr>
<td>07</td>
<td>3.2</td>
<td>0.1</td>
<td>9.9</td>
</tr>
<tr>
<td>08</td>
<td>0.0</td>
<td>0.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>
Use 100 ul per assay tube. Aliquot 500 ul of each in 12 x 75 glass tubes sufficient for 20 separate standard sets. Freeze at -20°C. Each standard set is stable for three months.

D. 125I-insulin. Obtained from:

NEN, 1-800-551-2121
Insulin (Porcine), TyrA14, (125I), Receptor Grade (220 uCi/mmole), Cat.# NEX-196, $348.00/10ul

Use the radioactive material only in Room 105-111 in the limited area. Fill the record every time when tracer is used. Use 30,000 CPM/100 ul per tube. Dilute sufficient label for one month use. Aliquot and freeze at -20°C.

E. Normal Guinea Pig Serum Carrier (NGPS). Obtain from:

Pel Freez 1-800-643-3426
Box 68, Rogers, Ark. 72757
Cat.# 38119-3, $24.00/30 ml

Aliquot and store at -20°C. Stable until expiration date. Dilute 1-70 with assay buffer containing 3% PEG. Use 100ul per tube

F. Second Antibody (Goat Anti Guinea Pig IgG Serum). Obtain from:

Lincor Research
$60.00/1,000 tubes.

For lot GP2012, dilute the content of the vial with 10 ml of assay buffer. Aliquot and store at -20°C. For use, dilute to 1:10 with buffer containing 3% PEG and add 100 ul per tube.

EQUIPMENT:

Beckman J-6 Refrigerated Centrifuge

Day 1.

1. Pipet 100 ul of standards, quality controls and samples. Totals, blanks, standards, and quality controls are run in triplicate; patient samples are run in duplicate (see summary table at end of procedure.)

2. Pipet 300 ul of insulin buffer to the blank tubes (4-6) and 100 ul of insulin buffer to standards, quality controls and patients.

3. Pipet 100 ul of 1st antibody to tubes standards, controls and patient samples.
4. Pipet 100μl of Tracer to all tubes.
5. Vortex, except for totals, cover and incubate overnight at 4°C.

Day 2.

6. Add 100 μl of 2nd antibody to all tubes (except totals).
7. Add 100 μl of NGPS (Normal Guinea Pig Serum Carrier) to all tubes (except totals).
8. Vortex, cover and incubate 2 hours at 4°C.
9. Add 1 ml of wash buffer (0.05 M phosphosaline, pH 7.5, no BSA, no PEG) to all tubes, except totals.
10. Vortex, centrifuge for 30 minutes at 3,500 RPM (2,500 X g).
11. Pour off supernatant and count pellet.

CALCULATION:

1. Average triplicate counts for blanks (tubes 1-3), total counts (tubes 4-6) and total binding (tubes 7-9), and average duplicates of remaining samples.

2. Subtract the average non-specific binding (NSB) counts (Blank tubes) from each average count (except for total counts). These are the counts used in the following calculations.

3. Calculate the percentage of tracer bound ((Total Binding Counts/Total Counts) X 100). This should be 34-40%.

4. Calculate the percentage of maximum binding (% B/B₀) for each standard and sample (%B/B₀ = (Sample or Standard/Total Binding) X 100).

5. Plot the % B/B₀ for each standard of the y-axis and known concentration of the standard on the x-axis. The use of log-log graph paper or the use of the Stats Graphics Linear Regression Program will result in a nearly linear curve.

6. Determine the insulin of the unknown samples (patients and controls) by interpolation of the reference curve.

LIMITS OF PROCEDURE:

1. Assay should be rejected if any 2 of the 6 reference controls falls outside of 2 SDS. See supervisor.
2. If the difference between duplicate results of a sample is > 10% CV, repeat the sample.

3. The limit of sensitivity for the insulin assay is 3.2 uU/ml.

4. The limit of linearity for the human insulin assay is 200 uU/ml. Any result greater than 200 uU/ml should be repeated on dilution using zero standard as a diluent.

**INTERPRETATIONS:**

**INSULIN STANDARDS**

To obtain human insulin standards from Eli Lilly and Company, write to:

Mr. Thomas Jeatran  
Registered Pharmacist  
Lilly Research Laboratory  
Lilly Corporate Center  
Indianapolis, IN 46285.

In the letter you have to write that insulin will be used only for research, not for humans. Mr. Jeatran will send you a form. It should be filled out and sent back to him. After the form is reviewed, he will send you the Reagent.

When insulin standards from other sources are used make sure that the conversion of uU of insulin to ng by the factor 25 uU/ng is performed.

6.3.5 Urinary Albumin

This assay is performed by the laboratory of Dr. Peter Bennett at the NIDDK Research Center in Phoenix, AZ, using an automated nephelometric immunochemical procedure. Light scattering units are measured during a reaction of albumin with a monospecific antibody to human albumin, using a Behring Nephelometer. Albumin concentrations are calculated by the instrument using a multipoint calibration curve constructed from a commercial calibrator which corresponds to WHO standards. Two normal and two abnormal controls are included in each run. At least 3 of the 4 controls must be within 10% of the established mean value for that control. Samples are run in duplicate or triplicate; coefficient of variation for replicates must be less than 7.07. The mean of two values or median of 3 values is reported.
6.3.6 Urine Creatinine

Urine creatinine is measured in the Phoenix NIDDK Laboratory using an automated alkaline picrate methodology run on an Alpkem Rapid Flow Analyzer. It is based on a procedure described by Chasson, Grady, and Stanley in 1961. Two aliquots of a normal and abnormal control are included at the beginning and end of each run. An assay is acceptable if at least 5 of the 8 controls are within 5% of the established mean and at least two of the within range values are for the low control and 2 are for the high control. Samples are run in duplicate or triplicate. Coefficient of variation for replicates must be less than 3.54. Mean of duplicates or median of triplicates is reported.

6.3.7 Fibrinogen: Functional Method (BBL Fibrometer)

This assay is contracted to the laboratory of Dr. Russ Tracy at the University of Vermont.

**Purpose:** The Dade method for determining fibrinogen concentration is based on the clotting time of citrated plasma using 100 NIH units/ml of thrombin (excess thrombin),

**Principle:** Fibrinogen, a soluble plasma protein, is converted to fibrin, an insoluble polymer of fibrinogen, in the presence of thrombin. Fibrinogen concentration determines the reaction rate using high concentrations of thrombin and relatively low concentrations of fibrinogen. The thrombin clotting time versus fibrinogen concentration is linear when plotted on log-log paper. Therefore, the longer the clotting time, the less the concentration of fibrinogen.

**Equipment:**
1. BBL Fibrometer
2. 0.9 ml fibrometer probe
3. 12 x 75 mm plastic tubes
4. 50 μl, 100 μl, 200 μl MLA pipettes, fibropipet
5. fibrometer reaction cups
6. disposable MLA and fibrotip pipette tips

**Reagents:**

**Stock Chemicals**
1. Bovine thrombin, topical Parke-Davis 10,000 NIH Units
2. Sodium Diethylbarbiturate (Na Barbital) NaC₈H₁₁N₂O₃ F.W. 206.18 Fisher Scientific B-22 500 gms
3. Sodium chloride (NaCl F.W. 58.44) Sigma
4. Concentrated Hydrochloric acid (HCl)
5. 85% Phosphoric Acid

**Stock Reagents**
1. 0.1 N HCL
   Slowly add 4.2 mls concentrated HCL to 250 mls Milli-Q water in a 500 ml volumetric flask. Fill to the 500 ml mark with Milli-Q water. Make fresh and use immediately.
2. Veronal Buffer
   Mix together:
   11.456 gm Na Barbital
   14.610 gm NaCl
   430 mls 0.1 N HCL (freshly made)
   Add these reagents to approximately 1000 mls Milli-Q water in a 2000 ml volumetric flask. When dissolved in about 1800 mls of distilled water, adjust pH to 7.35, fill to 2000 ml mark and recheck pH. Store at 4°C. Stability is 6 months.

3. Veronal Buffer with 5mM CaCl₂ (for use with EDTA plasma). Made same as #2 although 0.734 g/L CaCl₂ is also added to the solution.

4. 1% Phosphoric Acid
   Slowly add 11.7 mls of 85% phosphoric acid to approximately 500 mls distilled water in a 1 liter volumetric flask. Fill to 1000 ml mark with distilled water. Store at RT. Stable for one year.

5a. Standard Curve (citrated plasma)
   Dade Fibrinogen Calibration Reference is used by diluting 1 vial with 1.0 Milli-Q water then making dilutions as follows:
   
<table>
<thead>
<tr>
<th>Dilution</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>0.1 ml standard and 0.4 ml Veronal buffer.</td>
</tr>
<tr>
<td>1:15</td>
<td>0.1 ml standard and 1.4 ml Veronal buffer.</td>
</tr>
<tr>
<td>1:40</td>
<td>0.1 ml standard and 3.9 ml Veronal buffer.</td>
</tr>
</tbody>
</table>

   The dilutions are assayed on the fibrometer, in duplicate, and mean values (seconds) are recorded. This procedure is repeated twice more, preferably on two other work days, in order to have three different groups of values (seconds) for the three standard dilutions. Record in fibrinogen standard book. An average for each standard dilution is obtained (in seconds). There is an assay value on each vial of Dade Calibration Reference which may vary with each lot of standard. Each dilution has an assay value of fibrinogen concentration determined by the following:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>Assay value x 2</td>
</tr>
<tr>
<td>1:15</td>
<td>Assay value from 1:5 dilution divided by 3</td>
</tr>
<tr>
<td>1:40</td>
<td>Assay value divided by 4</td>
</tr>
</tbody>
</table>

   For each standard having a specific fibrinogen concentration there is a corresponding thrombin clotting time. Linear regression is used on TI-55III to establish a standard curve using the values obtained from assaying three different vials of Dade Calibration Reference: See TI 55III guidebook, pages 1-30.

5b. Standard Curve (EDTA plasma) (revised 9/15/89)
   For samples drawn into EDTA tubes, a standard curve using plasma treated with EDTA is made. A sample of citrated plasma obtained from lab tech, MK, was determined to have a fibrinogen level of 222 mg/dl in a
1:10 dilution as assayed on the fibrometer. Plasma obtained from an EDTA tube drawn on lab tech, MK, was also used. This plasma was run in 1:5, 1:10, 1:15, and 1:40 dilutions. Dilutions were made in Veronal Buffer with 5 mM CaCl₂. The dilutions were assayed on the fibrometer in duplicate, and mead values (seconds) were recorded. This procedure was repeated twice, one time each by individual lab techs, MK and DG. Values agreed among the different runs and a standard curve was made according the above instructions (5a).

6. Working Reagents
Thrombin (100 NIH U/ml) PREPARE USING PLASTIC BEAKERS, TUBES, AND PIPETTES.
Bovine Thrombin Topical, Parke-Davis, 100,000 NIH Units - Dissolve each vial of 10,000 NIH Unit thrombin in 100 mls of Veronal Buffer. Make 500 mls, aliquot 4 mls into labelled 5 ml 12 x 75 mm plastic tubes, cap with blue plastic caps, and place into plastic containers with lids. Store at -70°C.

7. Controls
Normal Plasma (N-IO pooled plasma from the Medical Center Hospital of Vermont) Ci-Trol Level I

8. Specimen
Recover sample to be assayed from freezer, thaw at 37°C, and place on ice.

Procedure: Turn on fibrometer(s) and heating block(s) to allow for at least a 20-minute warm-up time period. Lift the fibrometer probe into position and clean with 1% phosphoric acid. Rinse the electrodes well (x4) with distilled water.

Thaw the necessary number of working thrombin allquots required for the number of plasma samples being assayed. [i.e., with one 4 ml aliquot approximately 40 cups or 20 samples can be assayed.] After removing aliquots from the freezer, leave at RT for 5 - 10 minutes, then place into 37°C waterbath until completely thawed. Leave tubes at RT during assays.

Reconstitute 1 vial of Ci-Trol Level I with 1.0 ml Milli-Q water, swirl gently, and allow to equilibrate for 10-15 minutes. Place vial in ice bath with aliquots of thawed pooled plasma and patient’s plasmas.

Dilute pooled plasma, Ci-Trol Level I 1:10 using 0.1 ml sample add 0.9 ml Veronal Buffer. Also, make 1:90 dilutions of controls using 0.1 ml control and 2.9 mls Veronal Buffer. On EDTA plasma, patient samples are diluted 1:20 by pipetting 0.05 ml patient plasma in .95 ml Veronal buffer with 5 mM CaCl₂. This dilution was made no earlier than 5 minutes before addition of thrombin in the fibrinogen assay.

Each dilution is assayed in duplicate. Note: 50 µl of sample and 450 µl buffer may be used for 1:10 dilutions, if necessary.
Pipette 0.2 ml of control and patients' plasma dilutions into Fibrocups at timed intervals and incubate in the fibrometer heating block for at least 2 minutes but less than 5 minutes. Transfer fibrocups, one at a time, to the reaction well.

Pipette 0.1 ml Thrombin (100 NIH U/ml) into the first fibrocup being assayed, at the same time activating the fibrometer's timer.

Record the number of seconds registered on the fibrometer for each cup, averaging the duplicated and using a mean value from which to convert seconds to mg/dl fibrinogen concentration.

If the clotting time is shorter than the lowest value from the standard curve, dilute the EDTA plasma sample 1:30 using 0.1 ml sample and 2.9 ml Veronal Buffer with 5 mM CaCl₂ (or dilute the citrate plasma sample 1:20 using 50 µl sample and 950 µl Veronal Buffer). Assay in duplicate. Multiply the calculated fibrinogen concentration by 2 to correct for the 1:20 dilution. Plasma samples having low fibrinogen concentrations, such that no clot forms by the time given for the 1:40 standard (usually 35-40 seconds), are diluted 1:5 using 0.1 ml sample and 0.4 ml Veronal Buffer, assayed in duplicate. Calculated fibrinogen concentration is divided by 2. (For EDTA samples, dilute 1:10 using 0.1 ml sample and 0.9 ml Veronal Buffer with 5mM CaCl₂.) A dilution less than 1:5 is not usually performed since there is often insufficient volume of plasma sample.

Results: Average the duplicate times for each dilution and calculate the corresponding fibrinogen concentrations form the calculator programmed with the standard regression curve (or use conversion table when available). Report all values in mg/dl, including control values. Make sure all dilutions not 1:10 are corrected (i.e. multiply by 2 for 1:20, divide by 2 for 1:50).

Discussion 1. Fibrinogens are run at 37°C.
2. The thawed working thrombin is used at RT but placed on ice or refrigerated when not being used.
3. Controls are assayed at the beginning of each run, after every 12 dilutions, and at the end of each run. All control values must be plotted on Levy-Jennings graph and all 'out-of-control' values accounted for (i.e. whether a new dilution was made and assayed, whether original dilution was re-assayed, etc) and reported to the supervisor.

Quality control of Fibrinogen Assay

Control pools have been prepared by the laboratory and are assayed at the beginning for each run after 12 dilutions and at the end of each run. All control values are plotted on Levi-Jennings Graph. The assays are controlled using Westgard rules.
Graphs and all "out of control" values are accounted for and reported to the supervisor. Dr. Tracy's laboratory at the University of Vermont subscribes to the College of American Pathologists external quality control program and has consistently met their requirements. In addition, the laboratory at the University of Vermont has recently developed a standard, in collaboration with the ACP, for use in the standardization of fibrinogen assays.

6.3.8 Glycated Hemoglobin

HBA1c is measured in the Phoenix NIDDK laboratory by a variation of the cation exchange method which is adapted to the HPLC. Specially prepared hemolysate is injected into a small glass column fitted with cation exchange resin. A two buffer step gradient system is used. The first buffer having a higher pH and lower sodium ion concentration is pumped under moderate pressure to elute the fast fraction. The second buffer is pumped through the column to elute the main hemoglobin fraction. A spectrophotometer detection is used to continuously monitor the absorbance of the elute from 410 to 415 nm. Quantitation is accomplished by computing with an electronic integrator the area under the absorbance curve. For about 10% of the samples, where evidence of decomposition is seen, an affinity chromatographic method will be used as a backup.

Quality control is maintained using three levels of standards which are assayed at least once during each day's run. For the Strong Heart Study, in addition, quality control will be maintained by assaying blinded duplicates of 10% of the samples.

6.3.9 Glycated apoB

The percent of plasma apoB which is glycated is determined by solid-phase radioimmunoassay in the laboratory of Dr. Linda Curtiss of the Scripps Research Institute, La Jolla, CA.

THE TANDEM BEAD ASSAY FOR DETECTING GLUCOSYLATED LDL IN SERUM OR PLASMA

I. PREPARATION OF REAGENTS

A. Antibody Purification by Mono-Q Column Chromatography

1. Sample Preparation:
Clarity mouse monoclonal ascites fluid at 2,800 rpm in Beckman J6B centrifuge at 4°C for 10 min. Dialyze supernatant in 50,000 MWCO Spectra/Por 6 (Baxter) dialysis tubing against 10 mM Tris, 0.01% Na Azide, pH 8.0 at 4°C. Clarify the dialyzed ascites in 1.5 ml Eppendorf tubes at 13,250 rpm (11,600 xg) in a Beckman Microfuge 11 for 10 min. Filter the ascites with a prefector, then a 0.45 μ filter and finally a 0.2 μ filter.
2. Antibody Isolation:
   Mono-Q HR 16/10 column and FPLC system from Pharmacia.
   Start Buffer: 10 mM Tris, 0.01% Na azide, pH 8.0.
   Final Buffer: 0.5 M NaCl, 10 mM Tris, 0.01% Na azide, pH 8.0.
   Flow Rate: 6 ml/min.
   Chart Speed: 1 cm/min.
   Absorbance: A280; 0.2 AUFS
   Volume Injected: 1 ml of dialyzed and clarified ascites fluid.
   Peak Threshold: 2% of full-scale.
   Fraction Size: 1 min (6 ml) no delay.
   Gradient: Time 0 min -- inject sample.
             0-2 min -- start buffer.
             2-30 min -- 20% of final buffer to 60% of final buffer.
             30-34 min -- 100% of final buffer.
             34-38 min -- start buffer.

10 mM Tris, 0.01% Na azide, pH 8.0.
24.22 g Tris base, MW 121.1.
2.0 g Na azide.
   pH to 8.0 with more than 7 ml concentrated HCl.
   Bring volume up to 20 liters with nanopure water.
0.5 M NaCl, 10 mM Tris, 0.01% Na azide, pH 8.0.
58.44 g NaCl mw 58.44
   Bring volume up to 2 liters with 10 mM Tris, 0.01% Na azide, pH 8.0.

3. Antibody Detection and Concentration:
The antibody is detected with a solid-phase immunoassay where the antigen
is coated onto a microtiter plate, an aliquot of each column fraction is added
and allowed to incubate 1 hr at 37°C. After washing the mouse antibody is
detected with a radiolabeled antimouse Ig antibody. Fractions containing
antibody are pooled and concentrated in an ultrafiltration cell (Amicon)
with a 30,000 MWCO membrane. The concentrate is dialyzed in 50,000
MWCO Spectra/Por 6 dialysis tubing against PBS at 4°C and clarified.
Protein concentration is determined by the Modified Lowry Protein Assay.
Antibody purity is determined on a Pharmacia PhastGel.

B. Coupling of the APO B-Specific Antibody, MB47, to Sepharose 4B

150 mg of purified MB47 antibody in PBS is bound to 15 g of CNBr-activated
Sepharose 4B (Pharmacia 17-0430-01) as per manufacturer’s instructions. All
of the antibody is coupled to the Sepharose under these conditions.

C. Radioiodination of the Other APO B-Specific Antibody, MB24, and the
Glucitolysine-Specific Antibody, 8C11

200 μg of purified antibody is radiolabeled with 1 mCi of ¹²⁵I using six
Iodobeads (Pierce 28666) as per manufacturer’s instructions. The iodide is
removed by overnight dialysis at 4°C against PBS. The antibodies are
iodinated to specific activities of between 7,000 and 9,000 dpm/μg.
II. SAMPLE PREPARATION

A. NaBH₄ Reduction of the Plasma Sample

Measure plasma volume. To reduce the proteins add a volume of 1 M NaBH₄ in PBS to make the plasma 20 mM in NaBH₄. Incubate for 1 hr at room temperature, then 3 hr at 37°C. Dialyze in 12,000-14,000 MWCO Spectra/Por 2 (Baxter) dialysis tubing against 10 mM EDTA in PBS at 4°C. Determine the protein concentration with a Modified Lowry Protein Assay and store the sample at -70°C.

1 M NaBH₄ in PBS (1 M NaBH₄, 0.0005 N NaOH in PBS).
37.85 mg NaBH₄, MW 37.85.
Mix and add: 5 µl 1 N NaOH and 945 µl PBS, pH 7.2.

B. Protein Determination -- Modified Lowry

Standards: Duplicates of BSA containing 0-70 µg BSA.
Volume: 100 /µl of standard or sample. Use water to bring sample volume to 100 µl, if necessary.
Procedure: Mix 1 part Reagent B with 100 parts Reagent A.
Add 2 ml to standards and samples.
Add 200 µl of Folin Reagent diluted 1:2 in water.
Vortex immediately.
Let stand at room temperature for at least 45 min.
Read absorbance at 660 nm wavelength on plate reader.

Reagent A:
2% Na₂CO₃, 0.4% NaOH, 0.16% Na tartrate, 1% SDS.
Dissolve in the following order:
8 g NaOH mw 40.00
40 g Na₂CO₃ MW 105.99 (or 46.8 g Na₂CO₃·H₂O MW 124.00)
3.8 g Na tartrate MW 230.08 (Na₂C₄H₄O₆·2H₂O)
20 g SDS (Sodium dodecyl sulfate)
Bring volume up to 2 L with nanopure water.

Reagent B:
4% CuSO₄·5H₂O
4 g CuSO₄·5H₂O
Bring volume up to 100 ml with nanopure water.

Folin and Ciocalteau Reagent: SIGMA F9252.

III. THE TANDEM BEAD ASSAY FOR GLYCOSYLATED LDL

Wash the MB47 Sepharose 4B beads (MB47 antibody coupled to Sepharose 4B) twice with PBS/EDTA/BSA (1 mM EDTA, 3% BSA in PBS). Prepare a 10X volume/volume (buffer/beads) suspension in PBS/EDTA/BSA. Aliquot 200 µl of the suspension to a siliconized 12X75 glass tube. Clarify the stored NaBH₄-reduced plasma at 9,500 rpm (7.000 xg) for 5 min in a Beckman Microfuge 11 and add 10 µl of plasma or control to each of six tubes containing the bead suspension. Incubate overnight at 4°C on a rotating platform. Wash the beads twice with 3 ml of PBS/EDTA/BSA at 4°C by pelleting the beads at 700 rpm for 5 min in a Beckman J6B centrifuge at 4°C. Aspirate off the supernatant. Add 100 µl of 125I-8C11 antibody at 5 µg/ml to three tubes per plasma sample. Add 100 µl
of $^{125}$I-MB24 antibody at 0.1 $\mu$g/ml to the remaining three tubes per plasma sample. Incubate 4 hr at 4°C on a rotating platform. Wash twice with 3 ml of PBS/EDTA/BSA at 4°C. Pellet and aspirate as above. Count tubes in a gamma counter.

Calculate the trichloroacetic acid (TCA)-precipitable dpm per ng of $^{125}$I-radiolabeled antibody. To do this, subtract the PBS/EDTA/BSA buffer control mean cpm (assay zero control) from the plasma mean cpm. Divide this by the TCA-precipitable cpm per ng for each antibody. The result is the ng $^{125}$I-antibody bound by the immobilized plasma LDL. To obtain the ratio of glycosylated LDL to total LDL, divide the ng of $^{125}$I-8C11 bound by the ng of $^{125}$I-MB24 bound.

The antibody coupled beads can be regenerated by washing them with 3M KI until the $^{125}$I-cpm are removed. Regenerated beads can be reused in the assay.

6.3.10 LEUCOCYTE DNA ISOLATIONS

REAGENT PREPARATION

Stock Solution:
1. Autoclave DI-H$_2$O (10 x 500 ml) → for Reagent preparation
2. Buffer B: 3.85 g NH$_4$Cl in 500 ml DI-H$_2$O (from #1)
3. Buffer C: 0.198 g NH$_4$HCO$_3$ in 500 ml DI-H$_2$O (from #1)
4. Proteinase K comes in 100 mg lyophilized powder.
   - Add 10 ml DI-H$_2$O (from #1) into the vial to make 10 mg/ml Proteinase K working reagent.
   - Filter this 10 mg/ml Proteinase K
   - It is recommended to prepare a 30-40 ml batch of Proteinase K (10 mg/ml) and filter this batch.
   - Aliquot this Proteinase-K (10 mg/ml) into 5 ml portion → and freeze at -20°C.
5. Ethanol
6. 8-Hydroxyquinoline (Sigma MW = 145.2)
7. 2M Tris pH 7.51 (MW = 121.1); 60.55 g Tris/500 ml DI-H$_2$O → Adjust pH to 7.51
8. 0.2M Tris pH 7.51 → 1/10 dilution of #7
9. 0.5M EDTA pH 7.98 (MW = 372.2); 93.05 g/500 ml → Adjust pH to 7.98
10. 3M NaAc pH 5.21 (MW = 82.03); 123.05 g/500 ml → Adjust pH to 5.21
11. TE Buffer: 500λ 2M Tris pH 7.51 + 200λ 0.5 m EDTA pH 7.98 in 100 ml H$_2$O
12. TES Buffer2.5 ml 2M Tris pH 7.51
   1 ml 0.5 m EDTA pH 7.98) in 500 ml DI-H$_2$O
   2.5 g SDS)
13. Phenol (solid): Wear gloves and lab coat; fume hood
   Step I: 500 g Phenol+ 0.5 g Hydroxyquinoline in 500 ml of DI-H$_2$O → 60°C until phenol gets into solution. Stir O/N at 4°C to get a saturated Phenol in H$_2$O.
Step II: Pour #1 to 1000 ml separate funnel. There will be 2 layers:

→ DI-H₂O

→ Phenol saturated with H₂O

PREFERRED METHOD: Take reagent bottle, heat to 60°C → 500 ml liquid prepare as in #1. This prevents from weighing 500 g Phenol → It stinks!

Always save the yellow bottom layer. Collect this Phenol saturated H₂O in a 1000 ml beaker.

Step III: Add 500 ml of 0.2 M Tris pH 7.51 into Phenol saturated with H₂O → Stir at 4°C O/N.

Step IV: Pour reagent from Step III into a 1000 ml separate funnel. Again let it separate into 2 layers

→ 0.2 M Tris

→ Phenol saturated w/0.2 M Tris

Step V: Collect Phenol saturated with 0.2 M Tris in a 1000 ml beaker. Add 500 ml TES buffer into the beaker. Stir at RT O/N. Next a.m., pour this reagent into 1000 ml separate funnel. Collect the bottom yellow again (Phenol saturated with TES) into a 1000 ml reagent dispenser. Add another 500 ml TES into this Phenol saturated with TES. This is your final working Phenol solution. Store at 4°C.

→ TES

→ Phenol saturated w/TES

Excess Phenol saturated with TES → store at 4°C. It will remain 2 layers of liquid if things are done correctly.

14. Working Buffer B & C: 500 ml buffer + 50 ml Buffer C Prepare at least 2 liters.

WORKING PROTOCOL FOR LEUCOCYTE DNA PREPARATION

DAY 1 (wear gloves and lab coat)

1. RBC in purple tubes arrives at 650 Penn Medical Lab. Arrange it to be transferred to the Hyman Building with blue ice pack. Keep this at 4°C until processing time. Save SHS labels.

2. Log in samples in SHS log book. There will be a lab number and an SHS number. Save SHS labels.

3. Label 50 ml conicals with lab number. Label both the tube and cap.

4. Squirt buffer B & C into each purple tube. Cap it and invert 2 to 3 times and pour it into its corresponding conical tube. Each SHS patient usually has 3 purple tubes.
5. Q.S. each conical to 45 ml w/ B & C.
6. Cap tubes and shake vigorously.
7. Centrifuge these tubes at 3000 rpm @10'; 4°C (1st wash).
8. Discard upper liquid leaving compacted cells at the bottom. Be careful not to discard these cells at the bottom.
9. Q.S. Conical to 20 ml w/ B & C.
10. Centrifuge again at 3000 rpm @10'; 4°C (2nd wash).
11. Again discard the upper liquid. Pellet to be processed for DNA extraction should be observed by now at the bottom of the conical. Log-in those that have small amount of pellet.
12. Add 5 ml TES into each conical and also add 100λ Proteinase K (10 mg/ml) → vortex → incubate at 60°C O/N.

**DAY 2:**

13. Label a new set of conicals again.
14. Remove tubes from incubator and add 5 ml of working Phenol from a reagent dispenser
15. Vortex these conicals and centrifuge 3000 rpm @10'; 4°C.
16. Harvest the upper phase into the new set of conicals. Add 500λ 3m NaAc and 10 ml ETOH into each conical. Vortex. Sometimes insoluble DNA can be seen at this stage.

→ save

→ discard

17. Precipitate the DNA at -20°C O/N or longer.

**DAY 3 (final stage)**

18. Centrifuge conicals 3000 rpm @10'; 4°C.
19. Discard supernatant → DNA pellet at bottom (difficult to observe).
20. Invert these conicals to dry the tubes for at least 1 hour. It does not have to be totally dry.
21. While the tubes are drying, label 3x1.5 ml Eppendorf screw-capped vial per SHS patient w/SHS labels. These triplicate vials will be used to store the final DNA.
22. Add 300λ TE to each dry conical in order to resuspend the DNA pellet.
23. Add 20λ 3M NaAc to each Eppendorf vials. Add 100λ of resuspended DNA pellet into each vial.
24. Add 250λ ETOH into each vial. Insoluble DNA must be visible by now.
25. Cap these Eppendorf and sort the triplicates into 3 storage boxes.
26. Store these vials at -70°C.
27. Ship list with storage box # goes to office for data entry.
QUALITY CONTROL: (for every 10 tubes)

Pipet 2λ of DNA suspended in TE into 498λ of TE. Measure A260. Record the value in the log book.

For every 50 tubes assay DNA by agarose gel electrophoresis.

AGAROSE GEL ELECTROPHORESIS FOR THE EVALUATION OF DNA

The following protocol describes a horizontal electrophoresis of DNA on a low melting Agarose.

Stock Solutions

1. 70% ethanol and 100% ethanol
2. Proteinase K (10 mg/ml)
3. Phenol saturated with TES
4. Chloroform Isoamyl Alcohol (24:1)
5. 3M Sodium Acetate pH 5.21
6. Glycerol
7. 10% Bromophenol Blue (tracking dye)
8. 10% Xylene cyanol FF (tracking dye)
9. Tris Base
10. Boric acid
11. EDTA (mw: 372.2)
12. TE (Resuspending buffer)
13. Stock Buffers:

I. 10X TBE (Tris-Borate - EDTA Buffer)

 Prep: Tris-Base 432g
 Boric Acid 220g
 0.5M EDTA pH 7.98 160ml
 → Q.S. to 4L with DI-H2O

II. Gel Loading Buffer:

 Prep: (6 Fold; Store at 4°C)
 Glycerol 15 ml (Final concentration: 30%)
 10X TBE 5 ml
 Bromophenol Blue: 25ml of a 10.00% Bromophenol Blue
 Xylene Cyanol FF: 25ml of a 10.00% Xylene Cyanol FF
 (Final concentration of each dye is 0.25%)
 → Q.S. to 50ml with DI-H2O

III. Electrophoresis Running Buffer:

 1X TBE + Ethidium Bromide (final concentration is 0.5γ/ml)
14. 0.7% Agarose Gel in 500 ml 1X TBE → microwave 20 minutes at low temperature. This stock solution can be stored at RT and is reusable over and over again, provided there is no bacterial growth. Pour 100 mL of this 0.7% Agarose into a 100 ml storage bottle and use this as a working stock. Ethidium Bromide (Mutagen and Carcinogen! Be Careful. Wear Gloves and Lab Coat!)

15. HIND III - digested λDNA (from VWR/IBI) → molecular weight DNA marker.

**Preparation of a 50/ml marker:**

Example: The Hind-III marker (500λ) comes in with a concentration of 207λ/ml.

**Procedure:**

a. Centrifuge the whole 500λ Hind-III marker, 11000 rpm for 5 minutes
b. Mix the Hind-III marker by re-pipetting back and forth
c. Pipet 145λ of Hind-III and mix it with 355λ of TE and 100λ of Gel Loading Buffer
d. Final concentration is 50λ/ml

**PROCEDURE FOR AGAROSE GEL ELECTROPHORESIS**

**DAY 1: Preparation of Samples to be Loaded on Agarose Gel**

a. Thaw frozen vials containing DNA tuft. Transfer the DNA tuft into pre-labelled Eppendorf vials. For invisible DNA, centrifuge the vials (11000 rpm for 5 minutes) and discard the supernatant. The DNA pellet will be at the bottom of the vials.

b. To remove inorganic salt and rinse the DNA tuft, add 500λ of 70% Ethanol. Centrifuge 11000 rpm for 5 minutes → discard supernatant, invert the vials and air dry for 20 minutes.

c. Add 400 of TES to each vials and 50λ of Proteinase K (10 mg/mL) → vortex → centrifuge 11000 for 1 minutes.

   d. Resuspend the DNA by repeat pipetting and incubate the vials at 55°C for 1-hour.

   e. After 1-hour, add 400λ of Phenol saturated with TES → vortex → Centrifuge 11000 rpm for 2 minutes.

   f. Transfer the upper phase only to a new set of Eppendorf vials. Do not contaminate this DNA containing upper phase with proteins from the intermediate phase.

   g. Add 400λ CHCl₃ Isoamyl alcohol (24:1) into the vials → vortex → centrifuge for 2 minutes.

   h. Again, transfer the upper phase into a new set of Eppendorf vials. Do not transfer the lower phase CHCl₃.

   i. Add 40λ 3M NaOAc and 880λ 100% Ethanol into each vial.

   j. Store the vials at -70°C.
k. Thaw the frozen vials (j) for 10 minutes centrifuge 11000 rpm for 5 minutes.
l. Wash the DNA by adding 500λ of 70% Ethanol → centrifuge 11000 rpm for 5 minutes → DNA pellet will be visible at the bottom of the vials. If DNA pellet is invisible (for small concentration of DNA) → re-centrifuge for another 5 minutes and proceed to the next step.
m. Discard supernatant and invert the vials. Air dry the vials for 20 minutes.
n. Resuspend the DNA pellet in 100λ of sterile water.
o. To determine the concentration of DNA, pipet 2λ of suspended DNA (from n) into 498λ of sterile H₂O. Measure A₂₆₀. Calculation of DNA concentration:

\[
\frac{\gamma/\lambda}{OD_{260} \times 250}{20}
\]

DAY 2: Preparation of Agarose Gel Beds for Horizontal Electrophoresis

1. Have the Bio-Rad mini-gel electrophoresis apparatus set clean and ready.
2. Tape the plastic template all around with autoclave tape and fold the other half of the tape underneath the plate to form a mold into which the 0.7% Agarose Gel can be decanted into.
3. Insert the serrated plastic comb into its corresponding grooves (located close to the cathode). Normally, 14 sample wells are formed with the plastic comb.
4. Proceed to pour the melted 0.7% Agarose Gel (microwave the 100 mL stock 3 minutes at high temperature) into the above plastic mold.
5. Once the Agarose has solidified, proceed to remove tape gently as to not tear the agarose off its template. Remove the serrated plastic comb.
6. Insert the plastic tray into the gel cassette.
7. Pour Running Buffer (with Ethidium Bromide in it) into the gel cassette so as to immerse the solidified Agarose.
8. Form an array of 2λ Gel Loading Buffer on a clean surface of parafilm (use the inside section). Be sure that the amount of 2λ gel loading buffer dispensed on the parafilm correspond to the amount of samples prepared.
9. Pipet 1λ of the prepared DNA samples (n. above) and mix it with the 2λ loading buffer (from #8 above) on the parafilm surface and load the samples into its sample wells. Do this slowly and steadily to prevent puncturing of the gel bed. Also, try to avoid releasing the samples outside of the sample wells.
10. The extreme left sample well is usually allotted for the Hind-III DNA markers. Add 4A of the Hind-III marker straight into its slot (without mixing with loading buffer).
11. Be sure that the electrophoretic apparatus is connected properly to the power supply. Turn on the power and the running voltage is usually 50V for 20 minutes.
Photography of DNA

Ethidium Bromide is an intercalating agent between stacked bases and fluoresces on UV illumination.

Procedure:

Once the electrophoresis is over, remove the whole plastic tray (#6 above) and put it on top of the transilluminator (Model UVT 750-M/IBI).

Reminder: Put on anti UV goggles while viewing the illuminated gel. THIS IS A MUST.

Undergraded DNA will show only a single band. Proceed to take a Polaroid (#667) picture of the gel. Label the picture and put it in the record book.

NB: For QC purposes:
  a. $A_{260}$ measurement is performed on every 10th sample.
  b. Agarose gel electrophoresis is done on every 25th sample.

6.4 QUALITY ASSURANCE PROGRAM

In addition to the use of quality controls with each run, there is a need to assure that all the steps from blood drawing to sample receiving and laboratory measurement are correct, replication of unknown samples will be necessary.

At the discretion of the individual PI, one participant the first clinic day every week will serve as the replicate control - the recommended scheme is that should be the first participant who has a GTT for blood drawing that clinic day. If there is more than one clinic site, they should be rotated so that QA samples are sent from all clinic sites with the same frequency. A check list for blood drawing for quality control sample is available (Appendix 20b). A total of one urine tube and four extra blood tubes will have to be drawn, including:

Fasting: 2 [10-ml] Lavender top tube  
1 [14-ml] urine tube

2-Hour: 1 [5-ml] Lavender top tube  
1 [3-ml] Gray top tube

These are indicated on the Check List. One [10-ml] QA Lavender top tube should be processed and fresh plasma transferred to 5 [2-ml] cryovials with the proper ID label. Plasma from the second [10-ml] QA Lavender top tube should be placed in a screw top tube (do not freeze). The QA Gray top tube should be also processed as any regular Gray top tube and the plasma placed into two [2-ml] cryovials with red top. These 7 [2-ml] cryovials and the [14-ml] urine tube will be kept frozen in a separate zip-lock bag and sent in the next Dry-Ice shipment. The [5-ml] QA Lavender top tube taken at 2-hour and the screw top tube containing plasma should be placed in a separate zip-lock bag and sent directly to the Core Laboratory with the next shipment on Blue-Ice.
The numbering system for these quality control samples is similar to the Study ID and consists of 6 digits with the first digit corresponding to the center (1-SD, 2-OK, 3-AZ), the second digit will be a “3” to indicate that the sample is a QA and a 4-digit sequence number. A complete set of tubes should not be expected for QA samples. The Coordinating Center should receive at monthly intervals the list matching Study ID to QA for analysis (Appendix 27b). This list should never be made available to the Core Laboratory. (Appendix 27b).

6.5 SAMPLE HANDLING AND STORAGE AT THE CORE LABORATORY.

6.5.1 Sample Handling for Refrigerated Shipment

The following procedure will be instituted for shipment sent on Blue Ice from the various clinics.

1. Shipping Box is received
2. Check off the enclosed Shipping List making sure that ID’s on the tubes matched with the ID’s on the Shipping List.

There should be a separate zip-lock bag for each subject containing the following tubes:

* 1 [14-ml] plasma tube
* 1 [5-ml] Lavender top tube
* 3 [10-ml] Lavender top tubes containing red cells and buffy coat

In addition a set of extra self-sticking pre-printed ID# corresponding to the samples sent should also be included in the box.

3. Gently mix the Lavender top tube by inversion and divide the content into 2 [2-ml] Cryovials pre-labeled with the ID#.
4. Place one of the cryovials into the Freezer Box labeled “HbA1c” which will be assayed when two boxes are filled - approximately 180-200 samples. Enter the ID of the sample on the HbA1c Log List.
5. Place the remaining cryovials into the storage freezer box labeled “Hb” making sure that the Seq Number of the storage box is recorded on the Shipping List received from the clinics.
6. Set up the plasma samples for lipid measurements and ultracentrifugation.
7. Send the 3 [10 ml] Lavender top tubes (red cells and buffy coat) to Molecular Genetics Laboratory at Medlantic for DNA isolation.
6.5.2 Procedure for Sorting and Logging Frozen Specimens

1. Frozen samples will arrive in containers (#1) with dry ice in it. Please observe the condition of the samples; is it frozen or thawed? If it is thawed, it should be reported to the Center of origin and a comment should be entered on the shipping list.

2. Have on hand a second insulated container large enough to hold two (2) cardboard freezer boxes (labelled Glucose and SHS ####), a third container large enough to hold one cardboard freezer box (labelled Urine) and a fourth container large enough to hold four (4) freezer boxes (labelled Insulin #, Fibrinogen #, Glycated LDL #, and Apo’s #).

3. Transfer some dry ice to all 3 insulated containers.

4. Have on hand the accompanying shipping list from the sender and SHS Log Book.

5. This process will require two (2) people, one to sort samples and the other to record SHS numbers and appropriate information on the logging slips.

6. Open the shipping container. Samples from each individual patient are stored in separate plastic bags. Each bag should contain 10 x 2 ml, 2 G-O, 2 G-2 and 1 urine vials. Except for QC samples (w/#3 on the second digit) which normally contain 5 x 2 ml plasma, 2 G-2 and 1 Urine vials.

7. Remove one plastic bag at a time from the shipping container and count the number of tubes including urine tubes. These should be checked off by patient number on the shipping list by the second person. After being checked off, the sorting process will begin. Any discrepancies should be recorded on the shipping list.

8. Sorting Process: Remove the vials one by one from each bag. Place the urine vial in the urine box, one set of G-O (yellow caps) and G-2 (red caps) into the Glucose box, four plasma vials into the four separate boxes in the fourth container. Place the remaining 6 x 2 ml plasma and one set G-O and G-2 into the Storing box. This storing box should have the prefix of the Center and the box numbers written on it. (SHS ####)

   PHX = Phoenix  
   OK = Oklahoma  
   SD = South Dakota

9. Repeat this process for each of the bags and be sure to record any missing vials on the Glucose list and the accompanying shipping list. QC samples (w/#3 as the second digit of the SHS numbers) should be placed in the fourth container (w/the four boxes) for later sorting.

10. After all the bags are processed and contents transferred to the appropriate cardboard freezer boxes, the QC samples can be processed.
11. Protocol for Sorting QC-samples:
   a. Urine tube - this is to be added to the urine box to be shipped with the regular samples to Phoenix.
   b. Two (2) vials (G-2 with red tops) - one of these is to be added to Edna’s regular glucose box; one of these is to be added to a box labelled “QC Samples XXX”.
   c. Five vials of plasma - one is to be added to the box for fibrinogen samples to be shipped with the regular samples to Vermont; one is to be added to the box for the glycated LDL samples to be sent with the regular samples to California; one is to be added to the box of insulin samples to be assayed by Kazumi; two (2) are to be added to the box labelled “QC Samples XXX”.

Therefore, the QC box will contain for each subject: three (3) vials - one (1) red top G-2 and two (2) regular plasma vials.

12. When the sorting is complete, store the cardboard boxes on their appropriate shelf in a -70°C freezer.

13. Glucose box, apo’s box and QC box should be placed on the bottom shelf while awaiting transfer to 650 Penn for assays.

14. Fibrinogen, glycated LDL and urine should also be placed on the bottom shelf while waiting to be shipped out for assays. The insulin box will be processed by Kazumi.

15. At this point, sample processing complete.

16. Final paperwork: Log in the sample #’s from the glucose list into an empty SHS list. Once this is done, xerox five (5) copies of the new SHS list and label it for urine, insulin, fibrinogen, glycated LDL and apo’s. Enter QC numbers on the QC list. File these lists in the SHS log book.

17. Be sure to send back the original shipping container to the appropriate center.

18. Sample logs go to office for entry onto dataset.

6.5.3 Procedure for Shipping Frozen Samples for Analysis

1. Shipping out of samples will be done only on Tuesday (especially for Urine samples to Phoenix, AZ) of the week.

2. When 2 to 3 boxes for each type of analyte are available, then those boxes can be shipped for analyses.

3. Place the 2 to 3 boxes of urine, etc. into each separate insulated container with dry ice.
4. Inside each insulated container, there will be a list of samples to be analyzed, two(2) address labels of the receiving center (with telephone numbers of MRF and the receiving center on it, THIS IS A MUST!).

5. Seal the container tightly with strong sealing tape.

6. Fill out the appropriate Federal express form, insert it inside the mailing pouch and attach it to the top of the container.

7. Place another address label (with phone number) on the side of the container.

8. Use a marker and write the following instructions on the box:

   MEDICAL SUPPLY  ORMA
   DRY-ICE _____ lbs.  UN 1845

9. Once this is done, store these boxes in a -20°C freezer (2nd floor) or -70°C if it’s available.

10. Call Federal Express (953-3333) and tell them you have a package pick up. FedEx will require an account number, weight of boxes to be picked up and zip code of receiving center. The destination of receiving centers are as follows:

    Urine: Ms. Linda Phillips
             NIH, STRONG HEART STUDY
             1550 E. Indian School Road
             Phoenix, AZ 85014
             602-263-1615

    Gylc LDL: Dr. Linda Curtiss
                Dept. of Immunology
                Research Institute of Scripps Clinic
                10666 N. Torrey Pines Road
                La Jolla, CA 92037
                609-551-8248

    Fibrinogen: Russell Tracy, Ph.D.
                  Department of Pathology
                  University of Vermont
                  104 Southwick Street
                  Burlington, VT 05405
                  802-656-0396

11. FedEx will give you a confirmation number. Usually, ADWA xxxx. Write this number on the box.

12. FedEx will come and pick up the boxes before 5 pm of the same day.

13. Call the appropriate receiving center and notify them of the impending shipment.
14. Glucose, Apo’s and QC boxes are also packed in dry ice and transported to 650 PML for analysis by anyone who is going down there.

6.6 INFECTION CONTROL POLICY

The Strong Heart Study recognizes that some study subjects might possibly be infected with Hepatitis virus, HIV virus or other infectious disease which might potentially be transmittable to either clinical personnel or laboratory personnel handling blood specimens. However, it is not feasible in a field study to pretest individuals before the examination, nor should they properly be excluded if a random sample of the population is desired. Since contact with individuals with infectious diseases can pose risk to study personnel, a policy has been developed for both clinical personnel during patient contact and also laboratory personnel handling blood specimens. These policies are attached to appendix 38.