Helena Lipoprotein Electrophoresis Procedure

INTENDED USE

The Helena Lipoprotein Electrophoresis Method is intended for separation and quantitation of plasma lipoproteins by cellulose acetate electrophoresis.

For In Vitro Diagnostic use.

Rx Only

SUMMARY

Since Fredrickson and Lees proposed a system for phenotyping hyperlipoproteinemia in 1965[1], the concept of coronary artery disease detection and prevention utilizing lipoprotein electrophoresis has become a reality.

Epidemiologic studies have related dietary intake of fats, especially cholesterol, and elevated blood levels of lipids with the incidence of atherosclerosis, the major manifestations of which are cardiovascular disease and stroke. Ischemic heart disease has also been related to hypercholesterolemia.[2, 3]

The need for accurate determination of lipoprotein phenotypes resulted from the recognition that hyperlipoproteinemia is symptomatic of a group of disorders dissimilar in clinical features, prognosis and responsiveness to treatment. Since treatments of the disorders vary with the different phenotypes, it is absolutely necessary that the correct phenotype be established before therapy is begun.[4]

In the classification system proposed by Fredrickson and Lees, only types II, III and IV have a proven relationship to atherosclerosis.

Plasma lipids do not circulate freely in the plasma, but are transported bound to protein and can thus be classified as lipoproteins. The various fractions are made of different combinations of protein, cholesterol, glycerides, cholesterol esters, phosphatides and free fatty acids.[5]

Several techniques have been employed to separate the plasma lipoproteins, including ultracentrifugation, thin layer chromatography, immunological techniques, and electrophoresis.

Electrophoresis and ultracentrifugation are two of the most widely used methods and each has given rise to its own terminology. Table I shows the correlation of these classifications and the relative lipid and protein composition of each fraction.

Table 1: Classification and Composition of Lipoprotein Fractions.

Classification according to:		Composition - % in each fraction			
Electrophoretic Mobility	Ultracentri-fuge	Protein	Glyceride	Cholesterol	Phospholipids
Chylomicrons		2%	98%		
Beta	LDL*	21%	12%	45%	22%
pre-Beta	VLDL*	10%	55%	13%	22%
Alpha	HDL*	50%	6%	18%	26%

*Non standard abbreviations: LDL (low density lipoprotein), VLDL (very low-density lipoprotein), HDL (high density lipoprotein).

Various exceptions to the above classifications inevitably exist. One of these is the “sinking pre-beta”, which is pre-beta migrating material which “sinks” in the ultracentrifuge along with the LDL (beta migrating) fraction.[6] This is the Lp(a) lipoprotein reported by Dahlen.[7] It is considered a normal variant found in 10% of the population.

Another exception is the “floating beta”, which is migrating material “floating” in the ultracentrifuge with the VLDL. This abnormal lipoprotein appears in Type III hyperlipoproteinemias. Various types of support media have been used for the electrophoretic separation of lipoproteins. Fredrickson originally used paper electrophoresis when devising his classification system.[1, 8] Other media that have been employed are agarose gel, starch block and polyacrylamide gel.[5, 7] Cellulose acetate is rapid and easy to handle, with a single plate holding up to 12 samples. It does not require expensive, complicated and bulky equipment, and it is readily adaptable to broad scale screening programs.

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PRINCIPLE

The specimen is applied to a cellulose acetate plate which has been presoaked in a tris-barbital buffer at pH 8.8. The lipoprotein fractions are separated by electrophoresis and then stained with a methanol solution of Fat Red 7B at an alkaline pH. The stained bands may be visually inspected for qualitative results or may be quantitated in a scanning densitometer using a 525 nm filter.

REAGENTS

1. **Electra HR Buffer (Cat. No. 5805)**

   Ingredients: Electra HR Buffer is a tris-barbital-sodium barbital buffer.

   WARNING: FOR IN-VITRO DIAGNOSTIC USE. DO NOT INGEST. HARMFUL IF SWALLOWED. The buffer contains barbital, which in sufficient quantity, can be toxic.

   Preparation for Use: Dissolve one package in 650 mL of deionized water. The buffer is ready for use when all material is completely dissolved.

   Storage and Stability: The packaged and dissolved buffers should be stored tightly closed at room temperature (15 to 30°C). Packaged buffer is stable until the expiration date on the package. Dissolved buffer can be stored for two months at 15 to 30°C.

   Signs of Deterioration: Discard packaged buffer if the material shows signs of dampness or discoloration. Discard dissolved buffer if it becomes turbid.

2. **Lipoprotein Stain (Cat. No. 5322)**

   Ingredients: Each bottle contains 0.28% (w/v) Fat Red 7B after dissolution in methanol.

   WARNING: FOR IN-VITRO DIAGNOSTIC USE.

   Preparation for Use: Prepare the stock stain solution at least 24 hours before use. Dissolve stain in 1 L methanol and stir for 24 hours. Allow stain to set for 24 hours and filter it.

   Storage and Stability: The reagent is stable until the expiration date on the bottle when stored tightly capped at 15 to 30°C. The stock stain is stable for 2 years at 15 to 30°C.

   Signs of Deterioration: Discard the stain if a large amount of precipitate forms. A small amount of precipitate is normal.

3. **Titan Lipo Plates (Cat. No. 3900)**

   Ingredients: Cellulose acetate plates.

   WARNING: FOR IN-VITRO DIAGNOSTIC USE.

   Preparation for Use: The plates are ready for use as packaged.

   Storage and Stability: The plates should be stored at 15 to 30°C and are stable indefinitely.

INSTRUMENTS

Any high-quality scanning densitometer capable of accurately scanning uncleared cellulose acetate on a backing at a wavelength of 525 nm may be used. Recommended is the Helena EDC (Cat. No. 1375) densitometer.

SPECIMEN COLLECTION AND HANDLING

Specimen: Serum or plasma from samples collected in EDTA may be used. Do not use plasma collected in heparin.

Patient Preparation: For the most accurate phenotyping of lipoprotein patterns, the following precautions should be observed before sampling:[9]

1. Discontinue all drugs, if possible, for 3-4 weeks.
2. The patient should be maintaining a standard weight, and on a diet considered normal, for at least one week.
3. Wait 4-8 weeks after a myocardial infarction or similar traumatic episode.
4. The patient should be fasting for a 12-14 hour period. Chylomicrons normally appear in the blood 2-10 hours after a meal; therefore, a 12-14 hour fast is necessary to define hyperlipoproteinemia.

Interfering Substances: Heparin therapy causes activation of lipoprotein lipase, which increases the relative migration rates of the fractions, especially the beta lipoprotein.[10]

Serum Storage: For best results, fresh serum should be used.

Storage at 2 to 8°C for no more than 5 days yields satisfactory results. Prolonged storage increases the migration rate of the pre-beta fraction. Do not freeze.[11]

PROCEDURE

Materials Provided: The following materials are needed for the Helena Lipoprotein Electrophoresis Procedure.

Item	
Hardware	**Cat. No.**
Super Z-12 Applicator	4090
Super Z-12 Sample Well Plate (2)	4096
Super CK Aligning Base	4094
Super Z Applicator	4084
Super Z Sample Well Plate (2)	4085
Super Z Aligning Base	4086
Zip Zone® Applicator	4080
Zip Zone® Sample Well Plate	4081
Zip Zone® Aligning Base	4082
5 µL Microdispenser and Tubes	6008
1000 Staining Set	5122
Titan III Carrying Rack	5110
Bufferizer	5093
Coolant Sponges	5045
Consumables	**Cat. No.**
Titan® III Lipo (60 mm x 76 mm)	3900
Electra® HR Buffer	5805
Lipotrol	5069
Lipoprotein Stain	5322
Blotters (89 mm x 108 mm)	5037
Blotters (76 mm x 102 mm)	5034
Lipoprotein Report Forms (1 pad)	5214
Zip Zone Prep	5090
Titan Plastic Envelopes (large)	5053
Titan Plastic Envelopes (small)	5052
Helena Marker	5000
Zip Zone® Chamber Wicks	5081
Glue Stick	5002
Quality Control Chart	5109

Materials Needed But Not Provided:

1.0 N Sodium Hydroxide: 40 g NaOH pellets diluted to 1 liter with deionized water

Scanning densitometer with 525 nm filter. Helena EDC is recommended

Mechanical rotator (adjustable to slow speed)

Glass slides (of sufficient size to cover cellulose acetate plate)

Glycerine solution: Working solution = 3 parts glycerine to 1 part deionized water.

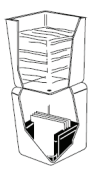
Working Stain Solution: Prepare the working stain solution immediately before use. Add 30 mL of stain to a shallow staining dish. Pipette 10 mL of 1.0 N sodium hydroxide into the stain while swirling the solution. A dark precipitate may begin to form, but will not affect the performance of the plate. The working stain should be discarded after staining one plate. Discard the stain if a large amount of precipitate forms. A small amount of precipitate is normal.

SUMMARY OF CONDITIONS

Plate	Titan III Lipo (Cat. No. 3900)
Buffer	Electra HR diluted to 650 mL
Soaking Time for Plate	15-20 minutes
Sample Size	5 pL
Application Point	Cathode
Number of Applications	2
Electrophoresis Time	25 minutes
Voltage	180 V
Staining Time	15-25 minutes
Filter Wavelength	525 nm

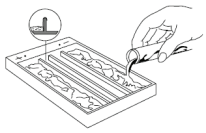
STEP-BY-STEP METHOD

A. Titan III Lipo Plate Preparation



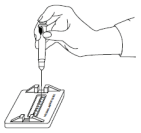
1. Dissolve one package of Electra HR Buffer in 650 mL deionized water.
2. Properly code the required number of Titan III Lipo Plates by marking on the glossy, hard side with a marker. It is suggested that the identification mark be placed in one corner so that it is always aligned with sample No. 1.
3. Soak the required number of plates in Electra HR buffer for 15-20 minutes. The plates should be soaked in the Bufferizer according to the instructions for use included with the Bufferizer. Alternately, the plates may be wetted by slowly and uniformly lowering a rack of plates into the HR Buffer. The same soaking buffer may be used for soaking up to 12 plates or for approximately one week if stored tightly closed. If used for a more prolonged period, residual solvents from the plate may build up in the buffer or evaporation may alter buffer concentration.

B. Electrophoresis Chamber Preparation

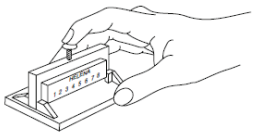


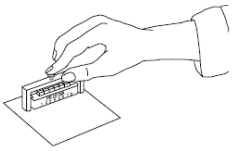
1. Pour approximately 100 mL of HR Buffer into each of the outer sections of the electrophoresis chamber.
2. Wet two disposable wicks in the buffer and drape one over each support bridge, making sure it makes contact with the buffer and that there are no air bubbles under the wicks.
3. Place two frozen coolant sponges into the center wells of the electrophoresis chamber.
4. Cover the chamber to prevent buffer evaporation. Discard electrophoresis buffer and wicks after use.

C. Sample Application

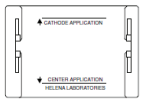


1. Fill each well of the sample plate with 5 µL of sample using the microdispenser. Cover the samples with a glass slide if they are not used within 2 minutes.
2. Prime the applicator by depressing the tips into the sample wells 3 or 4 times. Apply this loading to a piece of blotter paper. Priming the applicator makes the second loading much more uniform. Do not load applicator again at this point, but proceed quickly to the next step.

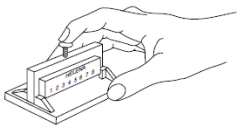


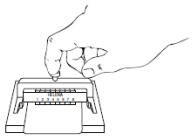


3. Remove the wetted Titan III Lipo Plate from the buffer with the fingertips and blot once firmly between two blotters. Before placing the plate in the aligning base, place a drop of water or buffer on the center of the aligning base. This prevents the plate from shifting during the superimposed application. Place the plate in the aligning base, cellulose acetate side up, aligning the top edge of the plate with the black scribe line marked "CATHODE APPLICATION". The identification mark should be aligned with sample No. 1.

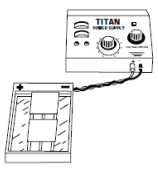


4. Apply the sample to the plate by depressing the applicator tips into the sample well 3 or 4 times and promptly transferring the applicator to the aligning base. Press the button down and hold it 5 seconds. Make a second super-imposed application by repeating this step.



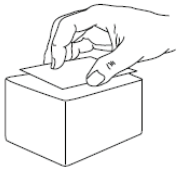


D. Electrophoresis



1. Quickly place the plate, cellulose acetate side down, in the electrophoresis chamber. The application point should be closer to the cathode (-). Place a weight (glass slide, etc.) on the plate to insure contact with the wicks. Cover the chamber securely.
2. Electrophorese the plate for 25 minutes at 180 volts. Power must be applied within 5 minutes after a plate has been placed in the chamber.

E. Staining and Evaluation of Lipoprotein Bands



1. Prepare the working staining solution approximately 5 minutes before the end of the electrophoresis time. Add 30 mL of stain to a shallow staining dish. Pipette 10 mL of 1.0 N sodium hydroxide into the stain while swirling the solution. A dark precipitate may begin to form but will not affect the performance of the plate. Prepare one dish for each plate. Cover the dish to reduce evaporation.
2. Remove the plate from the chamber at the end of the electrophoresis period, blot gently and immerse, cellulose acetate side up, in the staining solution.
3. Stain the plate for 15 to 25 minutes. It is recommended that the staining dish be placed on a serological rotator (slowest speed) during the staining process.
4. Remove the plate from the stain and wash it in tap water for a few seconds. If necessary, gently wipe off residual precipitate. If the plate is to be scanned in the densitometer, quickly proceed to the next step.
5. After rinsing away excess stain with water, carefully layer the plate, cellulose acetate side down, onto a glass slide, avoiding trapped air bubbles. Blot away excess water.
6. Scan the lipoprotein bands in a scanning densitometer using the 525 nm filter. Place plate in densitometer cellulose acetate side up with the alpha band to the left (scanned first). Instrument zeroed anodic to the alpha band.

F. Treatment of Plate for Qualitative Results and Permanent Storage

The following steps should be taken if the plate is to be visually inspected for qualitative results only or if permanent storage of the plate after densitometry is desired. Plates cannot be scanned accurately after treatment with glycerine; therefore, must be scanned prior to the steps outlined below:

1. Dip the plate, cellulose acetate side up, in a mixture of 3 parts glycerine and 1 part methanol.
2. Remove the plate from the glycerine solution and blot.
3. Place the plate, cellulose acetate side up, on a clean blotter until dry.
4. The plate can be stored for an indefinite period of time. The glycerine solution can be used several times if kept tightly closed.
5. Store in a Titan Plastic Envelope (Cat. No. 5052 or 5053).

Stability of End Product: Plates to be scanned in the densitometer for determination of the relative percentages of the bands must be scanned as soon as possible. Plates to be visually inspected for qualitative evaluation only may be kept an indefinite period of time after being processed with glycerine and as outlined above.

Calibration: A calibration curve is not necessary because relative concentration of the bands is the only parameter determined.

Quality Control: The Helena Lipotrol (Cat. No. 5069) verifies all phases of the procedure, and should be used on each plate run. The control should be used as a marker for location of the lipid bands and may also be quantitated to verify the accuracy of each run. Refer to the package insert, provided with the control, for assay values.

RESULTS

The Alpha-lipoprotein (HDL) band is the fastest moving fraction and is located closest to the anode. The Beta-lipoprotein (LDL) band is usually the most prominent fraction and is near the origin, migrating only slightly anodic to the point of application. The pre-Beta lipoprotein (VLDL) band migrates between Alpha and Beta-lipoprotein. The mobility of pre-Beta lipoproteins varies with the degree of resolution obtained, the type of pre-Beta present, and the percent of Beta present. Sometimes pre-Beta will be seen as a smear just ahead of the fraction. Other times it may be split into two or more fractions or may be lacking altogether. The integrity of the pre-Beta fraction decreases with sample age.

Chylomicrons, when present, stay at the point of application. In samples with very high levels of chylomicrons, there will appear to be a smear of material extending anodically from the point of applications to the beta band.

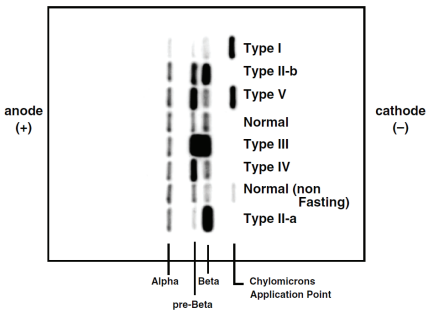


Figure 1: A Lipoprotein Plate showing the relative positions of the bands

Calculation of the Unknown:

Figure 2 is a typical lipoprotein scan. The relative percent of each band is computed and printed automatically by the EDC®. Refer to the Operators Manual for instructions. Calculating the mg/dL of total lipids from the relative percent values obtained is not recommended. (See LIMITATIONS)

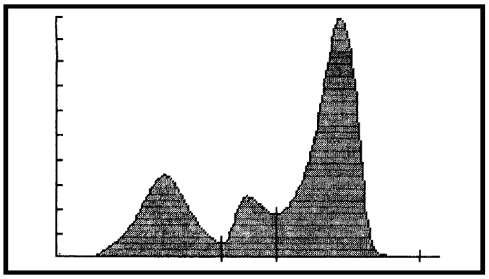


Figure 2: A typical lipoprotein scan produced by a Helena EDC

LIMITATIONS

Limiting Factors: Fat Red 7B, as well as the other Sudan fat stains, has a much greater affinity for triglycerides and cholesterol esters than it has for free cholesterol and phospholipids. Bands seen after staining with these dyes do not reflect a true quantitation of the total plasma lipids.[12] For this reason, it is not recommended that relative percentages of lipoprotein bands be used to calculate the total lipid content of each fraction based on a total plasma lipid value. Since most laboratories routinely offer total cholesterol and triglyceride levels, this information is unnecessary.

Interfering Factors: Specimens collected in heparin should not be used since heparin alters the migration patterns of the lipoprotein fractions.

Further Testing Required: Since the lipid composition of each lipoprotein fraction is variable, it is essential to determine total cholesterol and triglyceride levels before attempting to classify a pattern.[8, 9] When it becomes necessary to diagnose or rule out a Type III hyperlipoproteinemia, a more definitive quantitation of the lipoproteins such as ultracentrifugation[4] or electrophoresis on polyacrylamide gel[13] is essential.

REFERENCE VALUES

Normal Range:	Alpha:	23.1-54.5%
	pre-Beta:	10.1-35.9%
	Beta:	30.6-53.9%
	Chylomicrons	0-1.9%

These values were derived from an in-house study of apparently healthy fasting adults. Each laboratory should conduct its own normal range study because of population differences in various regions.

SPECIFIC PERFORMANCE CHARACTERISTICS

Precision

Within Run - A study was performed using a patient sample in replicate on one plate with the following variations.

n =12
SD =1.6
CV = 5.0%

Run to Run - A patient sample was tested in replicate on 5 plates with the following data.

n = 60
SD = 2.0
CV = 7.0%

INTERPRETATION OF RESULTS

LIPOPROTEIN PHENOTYPING USING THE HELENA LIPOPROTEIN ELECTROPHORESIS METHOD

Normal Pattern: A normal fasting serum can be defined as a clear serum with negligible chylomicrons and normal cholesterol and triglyceride levels. On electrophoresis, the Beta-lipoprotein appears as the major fraction, with the pre-Beta lipoprotein faint or absent and the Alpha band definite but less intense than the Beta.

Abnormal Patterns: A patient must have an elevated cholesterol or triglyceride to have hyperlipoproteinemia. The elevation must be determined to be primary or secondary to metabolic disorders such as hypothyroidism, obstructive jaundice, nephrotic syndrome, dysproteinemias or poorly controlled insulinopenic diabetes mellitus.

Primary lipidemia arises from genetically determined factors or environmental factors of unknown mechanism such as diet, alcohol intake, and drugs, especially estrogen or steroid hormones.[14] Also considered primary are those lipoproteinemias associated with ketosis-resistant diabetes, pancreatitis, and obesity. Diabetes mellitus and pancreatitis can be confusing, for it is often difficult to tell whether the hyperlipoproteinemia or the disease is the causative factor.8

PRIMARY LIPOPROTEINEMIAS:

The Fredrickson Classification

TYPE I: Hyperchylomicronemia

Criteria: Chylomicrons present, pre-Beta normal or only slightly elevated. Alpha and Beta decreased, often markedly so. Standing plasma with marked creamy layer.

Confirmation: A measurement of post-heparin lipolytic activity (PHLA) and the demonstration of severe intolerance to exogenous fat. The condition is rare and always familial. There has been no correlation to vascular disease. It is thought to be due to a genetic deficiency of lipoprotein lipase.[8]

TYPE II: Hyperbetalipoproteinemia

Criteria: Increased total cholesterol due to an increased Beta-lipoprotein cholesterol. Alpha cholesterol usually normal or low. Type IIa: normal pre-Beta, normal triglycerides, plasma clear.

Type IIb: increased pre-Beta and triglycerides, plasma clear to slightly turbid with no creamy layer.

This is one of the most common familial forms of hyperlipoproteinemias.

Secondary Causes: Myxedema, myelomas, macroglobulinemias, nephrosis, liver disease, excesses in dietary cholesterol and saturated fats.

TYPE III: "Broad Beta" - Abnormal Lipoprotein

Criteria: Presence of triglyceride burdened lipoprotein of abnormal composition and density. Cholesterol and triglyceride elevated. The abnormal material has broad beta electrophoretic mobility but separates with VLDL in the ultracentrifuge. Plasma is turbid to cloudy. The abnormal lipoprotein is also known as "floating Beta". The condition is rare.

Confirmation: Polyacrylamide gel electrophoresis[13] or ultracentrifuge studies to demonstrate the abnormal lipoprotein.

Type IV: Carbohydrate Induced and Endogenous Hypertriglyceridemia

Criteria: Increased pre-Beta, increased triglycerides, normal or slightly increased total cholesterol, Alpha and Beta lipoprotein usually normal. (An increased pre-Beta with normal triglyceride level is seen with the normal variant "sinking pre-Beta". Such samples do not belong to Type IV.)

Secondary causes: Nephrotic syndrome, diabetes mellitus, pancreatitis, glycogen storage disease, and other acute metabolism changes where mobilization of free fatty acids is increased. Endogenous triglycerides are very sensitive to alcohol intake, emotional stress, diet and changes in weight. Little effect is seen with exogenous triglyceride intake. Ninety percent of persons with familial Type IV have an abnormal glucose tolerance. Probably the most common type of hyperlipoproteinemia reflecting an imbalance in synthesis and clearance of endogenous triglycerides.

TYPE V: Mixed Triglyceridemia (Carbohydrate and fat induced)

Criteria: Increased exogenous and endogenous triglycerides, cholesterol increased, chylomicrons present, pre-Beta increased, Beta normal to slightly increased.

Secondary causes: Nephrosis, myxedema, diabetic acidosis, alcoholism, pancreatitis, glycogen storage disease and other acute metabolic processes.[4]

Note: Only Types II, III and IV has been correlated to vascular disease.

THE ALPHA LIPOPROTEINS IN DISEASE

Marked increase in the Alpha lipoproteins are seen in obstructive liver disease and cirrhosis. Marked decreases are seen in parenchymal liver disease. Tangier's disease is a rare genetic disorder characterized by the total absence of normal Alpha lipoproteins. Heterozygotes exhibit decreased levels of Alpha.[8] It should be noted that hyperestrogenemia (pregnancy and oral contraceptive use) may cause moderate elevations in the Alpha lipoproteins.[12]

DECREASES IN THE BETA LIPOPROTEINS

Abetalipoproteinemia is a primary inherited defect characterized by severe deficiency of all lipoproteins of density less than 1.063 (all but the Alpha lipoproteins). It is accompanied by numerous clinical symptoms and life expectancy is limited. A few cases of familial hypobetalipoproteinemia have been reported. There is some evidence that the mutation is different from that producing Abetalipoproteinemia.[8]

LIPOPROTEIN-X

Lipoprotein-X is an abnormal lipoprotein often seen in patients with obstructive liver disease. It consists of unesterified cholesterol, phospholipids and protein. It migrates slower than LDL. Because of its particular lipid contents, it stains poorly or not at all with the usual lipid stains and so is not usually detected by standard lipoprotein electrophoretic patterns.

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