SPIFE® 3000 Lipoprotein Procedure for Plastic Blades

Cat. No. 3340, 3341, 3342, 3343

INTENDED USE

The SPIFE Lipoprotein method is intended for the separation and quantitation of plasma lipoproteins by agarose gel electrophoresis using the SPIFE 3000 system.

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 relatively common. Epidemiologic studies have related dietary intake of fats, especially cholesterol, and blood levels of the lipids to 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. These 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 1 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	Ultra-Centrifuge	Protein	Glyceride	Cholesterol	Phospho-lipids
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 that “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 variant found in 20% of the population.[15] If a fourth band appears between pre-beta and alpha, it is Lp(a) and should be quantitated with pre-beta.

Another exception is the “floating beta,” which is beta migrating material “floating” in the ultracentrifuge with the VLDL. The abnormal lipoprotein appears in Type III hyperlipoproteinemias.

Various types of support media have been used for the electrophoretic separation of lipoproteins. When Fredrickson originally devised the classification system, he used paper electrophoresis.[1, 8] More recently agarose gel, starch block and polyacrylamide gel have been used.[5, 7]

PRINCIPLE

The specimen is applied to an agarose gel, the lipoprotein fractions are separated by electrophoresis and stained with Fat Red 7B. The stained bands may be visually inspected for qualitative results or may be quantitated in a scanning densitometer such as a QuickScan Touch Plus.

REAGENTS

1. **SPIFE Lipoprotein Gel**

   Ingredients: Each gel contains agarose in a sodium barbital buffer with EDTA, guanidine hydrochloride, bovine albumin, urea and magnesium chloride. Sodium azide and other preservatives have been added.

   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. The gel contains barbital which, in sufficient quantities, can be toxic. To prevent the formation of toxic vapors, this product should not be mixed with acidic solutions. When discarding this reagent always flush sink with copious quantities of water. This will prevent the formation of metallic azides which, when highly concentrated in metal plumbing, are potentially explosive. In addition to purging pipes with water, plumbing should occasionally be decontaminated with 10% NaOH.

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

   Storage and Stability: The gels should be stored horizontally at room temperature (15 to 30°C), in the protective packaging, and are stable until the expiration date indicated on the package. **DO NOT REFRIGERATE OR FREEZE THE GELS.**

   Signs of Deterioration: Any of the following conditions may indicate deterioration of the gel: (1) crystalline appearance indicating the agarose has been frozen, (2) cracking and peeling indicating drying of the agarose, (3) bacterial growth indicating contamination, (4) thinning of gel blocks.

2. **Lipoprotein Stain**

   Ingredients: When reconstituted as directed, the stain contains 0.1% (w/v) Fat Red 7B in 95% methanol.

   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY.

   Preparation of Stock Stain Solution: Dissolve the stain (entire contents of vial) with 1 L methanol. Stir overnight, allow stock solution to stand for 1 day and filter before use.

   Storage and Stability: The stain should be stored at 15 to 30°C and is stable until the expiration date indicated on the package. The diluted stain is stable for two months stored at 15 to 30°C. The filtered stain should be stored in a screw top container at 15 to 30°C.

   Signs of Deterioration: The diluted stain should be a homogeneous mixture free of precipitate.

INSTRUMENTS

A SPIFE 3000 instrument must be used to apply samples, electrophorese and dry the gel. The gel can be scanned on a densitometer such as the QuickScan Touch Plus (Cat. No. 1640). Refer to the appropriate Operator’s Manual for detailed operating instructions.

SPECIMEN COLLECTION AND HANDLING

Specimen: Serum or plasma from samples collected in EDTA may be used. Do not use plasma collected in heparin. Fresh serum is the specimen of choice.

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 needed for the procedure are contained in the SPIFE Lipoprotein Kits.

Sample Test Size	Cat. No.
80 sample	3340
60 sample	3341
40 sample	3342
20 sample	3343

SPIFE Lipoprotein Gels (10)
Lipoprotein Stain (1 vial)
REP Blotter C (10)
Electrode Blotter (20)
Blade Applicator Kit - 20 Sample

Materials provided, but not contained in the kit:

Item	Cat. No.
SPIFE 3000	1088
QuickScan Touch Plus	1640
Lipotrol	5069
REP Prep	3100
REP Gel Staining Dish (10)	1362
Gel Block Remover	1115
SPIFE Disposable Sample Cups (Deep Well)	3360
SPIFE 20-80 Dispo Cup Tray	3364
SPIFE Disposable Stainless Steel Electrodes	3388
Applicator Blade Weights	3387

Materials Needed but not Supplied:

Methanol

Destaining Solution: Mix 75 mL methanol with 25 mL deionized water.

STEP-BY-STEP METHOD

NOTE: If a SPIFE procedure requiring a stain has been run prior to running the Lipoprotein gels, the stainer unit must be cleaned/washed before drying the gel.

The new software version 1.20 has an automatic wash cycle prompted by initiation of a test which does not use the stainer unit for staining when the previous test did use the stainer for staining. To avoid delays after staining, this wash cycle should be initiated at least seven (7) minutes prior to the end of staining. To verify the status, press the **TEST SELECT/CONTINUE** button on the stainer until the appropriate test is selected. Place an empty Gel Holder in the stainer unit. If cleaning is required, the "Wash 1" prompt will appear, followed by "Plate out, Holder in" prompts. Press "Continue" to begin the stainer wash. The cleaning process will complete automatically in about 7 minutes. The unit is then ready to dry the gel.

I. Sample Preparation

1. If testing 61-80 samples, remove four Applicator Blades from the packaging. If testing fewer samples, remove the appropriate number of Applicator Blades from the packaging.
2. Place the four Applicator Blades into the vertical slots in the Applicator Assembly identified as 2, 6, 11 and 15. If using fewer Applicator Blades, place them into any of the four slots noted above.
   NOTE: The Applicator Blades will only fit into the slots one way; do not try to force the Applicator Blades into the slots.
3. Place an Applicator Blade Weight on top of each Applicator Blade. When placing the weight on the blades, position the weight with the thick side to the right.
4. Slide the appropriate number of cup strips into the slots in the Cup Tray.
5. Pipette 75-80 µL of patient serum or control into each well of the Disposable Cups. If testing less than 61 samples, pipette samples into the row of cups that corresponds with Applicator Blade placement. Cover the tray until ready to use.

II. Gel Preparation

1. Remove the gel from the protective packaging and discard overlay.
2. Place a REP Blotter C on the gel with the longer end parallel with the gel blocks. Gently blot the entire surface of the gel using light fingertip pressure on the blotter and remove the blotter.
3. Dispense approximately 2 mL of REP Prep onto the left side of the electrophoresis chamber.
4. Place the left edge of the gel over the REP Prep aligning the round hole on the left pin of the chamber. Gently lay the gel down on the REP Prep, starting from the left side and ending on the right side, fitting the obround hole over the right pin. Use lint-free tissue to wipe around the edges of the plastic gel backing, especially next to electrode posts, to remove excess REP Prep. Make sure no bubbles remain under the gel.
5. Thoroughly wash the electrodes with deionized water before and after each use. Wipe the carbon electrode with a lint-free tissue. The Disposable Stainless Steel Electrode must be patted dry because of the rough surface. Ensure that the endcaps are screwed on tightly. The Disposable Stainless Steel Electrode must be replaced after use on 50 gels. Unscrew the endcaps from the old electrode and screw them tightly onto the new electrode.
6. Place a carbon electrode on the outside ledge of the cathode gel block (left side of the gel) outside the magnetic posts. Improper contact between the electrode and the gel block may cause skewed patterns.
7. Place a Disposable Stainless Steel Electrode on the outside ledge of the anode gel block (right side of the gel) outside the magnetic posts.
8. Place an Electrode Blotter directly above and below the cathode end of the gel block. Slide the blotters under the ends of the carbon electrode so that they touch the gel block ends. Close the chamber lid.
9. Press the **TEST SELECT/CONTINUE** buttons located on the Electrophoresis and Stainer sides of the instrument until the **LIPO** option appears on the displays.

III. Electrophoresis Parameters

Using the instructions provided in the appropriate Operator's Manual, set up the parameters as follows for the SPIFE 3000:

Electrophoresis Unit

1) No Prompt Load Sample 1	00:02	20°C	SPD6	
2) No Prompt Load Sample 2	00:02	20°C	SPD6	
3) No Prompt Load Sample 3	00:02	20°C	SPD6	
4) No Prompt Load Sample 4	00:30	20°C	SPD6	
5) No Prompt Apply Sample 1	1:00	20°C	SPD6	LOC1
6) No Prompt Electrophoresis 1	20:00	16°C	400 Volt	150 mA
7) Remove gel blocks (continue) Dry 1	8:00	54°C		
8) No Prompt END OF TEST				

Stainer Unit

1) No Prompt Dry 1	10:00	70°C
2) No Prompt END OF TEST		

IV. Electrophoresis

1. Open the chamber lid. Place the Cup Tray with samples on the SPIFE 3000. Align the holes in the tray with the pins on the instrument.
2. With **LIPO** on the display, press the **START/STOP** button. An option to either begin the test or skip the operation will be presented. Press **START/STOP** to begin. The SPIFE 3000 will apply the samples, electrophorese and beep when completed.
3. Open the chamber lid, remove and dispose of Electrode Blotters. Dispose of blades and cups as biohazardous waste.
4. Using a Gel Block Remover, completely remove the two gel blocks from the gel and discard the gel blocks. Place an electrode on each end of the gel during the drying process to prevent curling. Close the chamber lid.
5. Press the **TEST SELECT/CONTINUE** button to dry the gel.

V. Visualization of the Lipoprotein Bands

1. **Preparation of Staining Solutions**
   a. **Stock Stain Solution:** Mix the Lipoprotein Stain in 1 L methanol. Allow to stir overnight and stand for one day. Filter before use. Store at 15 to 30°C.
   b. **Working Stain Solution:** Approximately 5 minutes before use, prepare a working solution of Lipoprotein Stain by adding 5 mL deionized water to 25 mL of stock Lipoprotein stain solution. For best results, add the water to the stain in a dropwise manner while swirling the solution.
   c. **Destaining Solution:** Mix 75 mL methanol with 25 mL deionized water. Mix thoroughly.
2. **Recommended Staining Procedure**
   a. Place the gel in the REP Gel Staining Dish, agarose side up. Carefully pour 30 mL of the Working Stain Solution directly on the agarose. Wait 4 minutes. Pour off the stain.
   b. Place the gel in Destain Solution for 10 to 20 seconds. Remove excess stain from the sample area using a gloved finger to gently and evenly wipe the gel surface. Pour off the Destain.
   c. Destain the gel again for 10 to 20 seconds. Pour off the Destain Solution.
   d. A brief final water wash may be necessary if trace amounts of background stain remain on the gel. Excessive destaining may cause light or faded bands and/or non-quantitation of control.
3. **Drying the Gel**
   a. Attach the gel to the SPIFE Gel holder by placing the round hole in the gel mylar over the left pin on the holder and the obround hole over the right pin on the holder.
   b. Place the Gel Holder with the attached gel facing backwards into the stainer chamber.
   c. With **LIPO** on the display, press the **START/STOP** button. An option to either begin the test or skip the operation will be presented. Press **START/STOP** to begin. The instrument will dry the gel.
   d. When the gel has completed the process, the instrument will beep. Remove the Gel Holder from the stainer and you can scan the bands.

VI. Evaluation and Quantitation

1. **Qualitative evaluation:** The SPIFE Lipoprotein Gel may be visually inspected for the presence of bands.
2. **Quantitative evaluation:** Scan the Lipoprotein Gel in the QuickScan Touch Plus using slit 5 and the Fat Red 7B filter.

Stability of End Product: Gels to be scanned for the quantitative determination of the bands must be scanned as soon as possible. Gels to be visually inspected for qualitative evaluation only may be kept an indefinite period of time after being processed.

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

Quality Control:

Lipotrol (Cat. No. 5069) can be used to verify all phases of the procedure and should be used on each gel 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 quantitations. Refer to the package insert provided with each control for assay values. Additional QC controls may be required for federal, state or local regulations.

REFERENCE VALUES

Reference range studies were established using 37 male and female adults with a total cholesterol of ≤ 200 mg/dL.

Lipoprotein Fraction	% of Total Lipoprotein
Alpha	12.6 - 46.6
Pre-Beta	0 - 57.1
Beta	21.7 - 67.7
Chylomicrons	< 1.0

Any quantitation of Lp(a) must be added to pre-beta for an accurate total pre-beta.

These values are intended as guidelines. Each laboratory should establish its own normal range study because of population differences in various regions.

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 cathodic to the point of application. The pre-beta lipoprotein (VLDL) band migrates between alpha and beta lipoprotein. The mobility of the pre-beta fraction 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 beta 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.

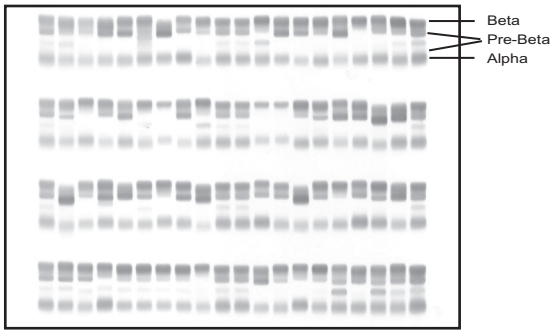

Figure 1: A SPIFE Lipoprotein gel illustrating the band positions.

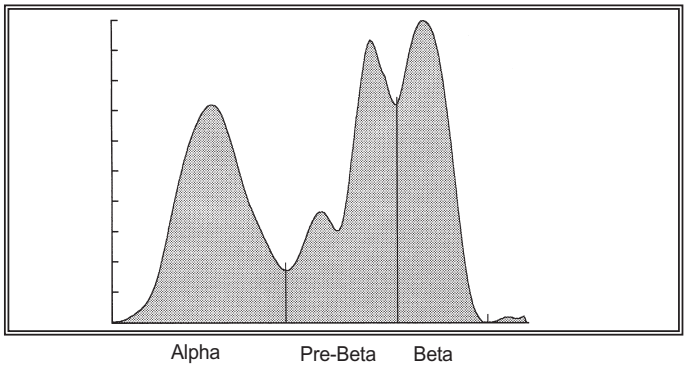

Figure 2: A scan of a SPIFE Lipoprotein pattern with an Lp(a) band.

Calculations of the Unknown: Figure 2 shows a typical lipoprotein scan. The relative percent of each band is computed and printed automatically by the densitometer. The calculated percent of the Lp(a) band must be added to the percent of the pre-beta band for a total pre-beta value.

Calculating the mg/dL of total lipids from the relative percent values obtained is not recommended. (See LIMITATIONS.)

INTERPRETATION OF RESULTS

Lipoprotein Phenotyping Using the SPIFE 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. If a fourth band appears between pre-beta and alpha, it is Lp(a) and should be quantitated with the pre-beta band.

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.

LIMITATIONS

Limiting Factors: Fat Red 7B, as well as the Sudan black 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 in each fraction from 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.

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 hyperproteinemias.

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 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 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 included)
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 have been correlated to vascular disease.

THE ALPHA LIPOPROTEINS IN DISEASE

Marked increases 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 lipoproteins.[8] It should be noted that hyperestrogenemia (pregnancy or 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.

LIPOPROTEIN-X

Lipoprotein-X is an abnormal lipoprotein often seen in patients with liver disease. It consists of unesterified (free) 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 methods.

PERFORMANCE CHARACTERISTICS

PRECISION

Within Run precision was evaluated by analyzing one sample 80 times on one gel. n = 80

Lipoprotein Fraction	Mean %	SD	CV
Alpha	25.2	0.8	3.1%
Pre-Beta	23.7	0.5	2.1%
Beta	51.1	0.9	1.7%

Between Run: Normal samples were run 80 times each on 5 gels with the following results. n = 400

Lipoprotein Fraction	Mean %	SD	CV
Alpha	24.1	2.1	8.5%
Pre-Beta	22.4	1.6	7.4%
Beta	53.5	3.5	6.5%

CORRELATION

Correlation studies were performed on 64 normal and abnormal specimens. The SPIFE Lipoprotein method was compared to the REP Lipo-30 method resulting in the following linear regression:

n = 64 X = REP Lipo-30
Y = 0.936X + 2.136 Y = SPIFE Lipoprotein
R = 0.957

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