SPIFE® Cholesterol Procedure

Cat. No. 3440, 3441, 3442, 3439, 3438

INTENDED USE

The SPIFE Cholesterol electrophoresis system is intended for use in the quantitative determination of cholesterol and cholesterol esters in the lipoproteins of serum using the SPIFE 3000 agarose electrophoresis system. The system is intended for the assessment of the cholesterol content of the high density lipoproteins (HDL), low density lipoproteins (LDL), very low density lipoproteins (VLDL), and Lp(a)-C, when present in concentrations greater than 2.5 mg/dL. However, in some patients Lp(a)-C may not be present at concentrations that are detectable by electrophoresis.
For In Vitro Diagnostic use.
Rx Only

SUMMARY

The relationship of HDL Cholesterol to coronary heart disease (CHD) was reported by Barr et al., 1951[1] and by Miller and Miller in 1975.[2] The work of Castelli et al.,[3-6] focused attention on HDL cholesterol assessment as the definitive laboratory test in determining the risk of coronary heart disease. The cholesterol content of the lipoprotein fractions has been determined by ultracentrifugation,[7] selective precipitation,[8] and electrophoresis on several media.[9] Clinical laboratory measurement of the serum lipoproteins is primarily due to their predictive association with risk of coronary heart disease (CHD). Current practice guiding laboratory measurement of total serum cholesterol, triglycerides, HDL cholesterol and LDL cholesterol is derived from recommendations of expert panels convened by the National Cholesterol Education Program (NCEP). The expert panels considered epidemiological, clinical and intervention studies in developing the recommendations for treatment decision cutpoints and recommended workup sequences for adults and children.
The clinical recommendations from the NCEP panels direct clinical laboratories to perform measurements of total, HDL and LDL, cholesterol and triglycerides. The triglycerides are primarily associated with chylomicrons, very low density (VLDL) and intermediate density (IDL) lipoproteins thought to be atherogenic, but the association of triglycerides with risk of coronary heart disease in epidemiological studies is ambiguous.
LDL, as the validated atherogenic lipoprotein based on its cholesterol content, is the primary basis for treatment decisions in the NCEP clinical guidelines.[10] The major protein component of LDL is apolipoprotein B100 (apoB) which has been measured previously by immunoassay. The common research method for accurate LDL cholesterol quantitation and the basis for the reference method is designated beta-quantification, beta referring to the electrophoretic term for LDL. The beta-quantification technique involves a combination of ultracentrifugation and chemical precipitation.[11,12] The beta-quantification method gives a so-called "broad cut" LDL which includes the Lp(a)-C lipoprotein,[13,14] often referred to as "lipoprotein little a".
The NCEP panel concluded that alternative methods are needed for routine diagnostic use, preferably ones which directly separate LDL for cholesterol quantitation.[15] One such direct method involves electrophoresis. Electrophoretic methods (reviewed in Lewis and Opplt[16,17]) have a long history of use in qualitative and quantitative analysis of lipoproteins. Electrophoresis not only allows separation and quantitation of major lipoprotein classes, but also provides a visual display useful in detecting unusual or variant patterns. Agarose has been the preferred media for separation of whole lipoproteins, providing a clear background and convenience.[18-21] Early electrophoretic methods were, in general, considered useful for qualitative analysis but less than desirable for lipoprotein quantitation because of poor precision and large systematic biases compared to other methods.[22] Recent improvements to the Helena SPIFE automated electrophoresis system demonstrate that electrophoretic quantitation can be precise and accurate. Evaluations demonstrate good separation of the major lipoprotein classes with precise and accurate quantitation of HDL, LDL, and VLDL cholesterol and Lp(a)-C in comparisons with the reference methods.[23]

PRINCIPLE

The SPIFE system separates the major lipoprotein classes using agarose electrophoresis. The lipoprotein bands are stained with enzymic reagent and their cholesterol content quantitated by densitometric scanning.

$$\text{Cholesterol Ester} \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol + Fatty Acid}$$

$$\text{Cholesterol + } NAD^+ \xrightarrow{\text{Cholesterol Dehydrogenase}} \text{Cholestenone + NADH + } H^+$$

$$\text{NADH + } H^+ \text{ + NBT} \xrightarrow{\text{Diaphorase}} NAD^+ \text{ + Formazan Dye}$$

The alpha band which migrates the farthest toward the anode corresponds to HDL. The next band, pre-beta, corresponds to VLDL, and the slowest moving beta band corresponds approximately to LDL. If a band appears between alpha and pre-beta, it should be quantitated as the Lp(a)-C band. This band may not be observed in every specimen. Chylomicrons, if present, remain at the origin. The amount of formazan dye produced is directly proportional to the amount of cholesterol and cholesterol esters originally present in the sample. The relative percent cholesterol in each fraction is obtained by scanning in a densitometer equipped with 570 nm filter or with the QuickScan Touch Plus Scanner.

REAGENTS

1. SPIFE Cholesterol 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. SPIFE Cholesterol Reagent

Ingredients: When reconstituted as directed, the concentration of the reactive ingredients is as follows:

Cholesterol Esterase (*Pseudomonas* sp.)	5.4 U/mL
Cholesterol Dehydrogenase (*Nocardia* sp.)	1.1 U/mL
Diaphorase (*Clostridium kluyveri*)	75.0 U/mL
NAD	35.3 mM
NBT	2.3 mM

Preparation for Use: Reconstitute each vial of SPIFE Cholesterol Reagent with 2.5 mL SPIFE Cholesterol Diluent. Swirl gently to dissolve. Do not shake. Be sure the reagent is completely dissolved before using.
Storage and Stability: Cholesterol Reagent should be stored at 2 to 8°C and is stable until the expiration date indicated on the vial. The reconstituted reagent is stable for 6 hours at 2 to 8°C.
Signs of Deterioration: The unreconstituted reagent should be uniformly pale or light yellow. The reconstituted reagent is a clear to light yellow solution.

3. SPIFE Cholesterol Diluent

Ingredients: Cholesterol Diluent contains 100 mM Hepes Buffer
Preparation for Use: The diluent is ready for use as packaged.
Storage and Stability: The diluent should be stored at 2 to 8°C and is stable until the expiration date indicated on the vial.
Signs of Deterioration: Discard the diluent if it shows signs of bacterial growth.

4. Citric Acid Destain

Ingredients: After dissolution, the destain contains 0.3% (w/v) citric acid.

WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. - IRRITANT - DO NOT INGEST.

Preparation for Use: Pour 11 L of deionized water into the Destain vat. Add the entire package of Destain. Mix well until completely dissolved.

Storage and Stability: Store the Destain at 15 to 30°C. It is stable until the expiration date on the package.

Signs of Deterioration: Discard if solution becomes cloudy.

INSTRUMENTS

A SPIFE 3000 must be used to electrophorese 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 samples are the specimen of choice.

Patient Preparation: The cholesterol content of the Alpha (HDL) and Beta (LDL) and Lp(a)-C lipoproteins is not materially affected by recent meals.[3] Therefore, if the HDL cholesterol is the only parameter of interest, the patient need not be fasting.

Interfering Substances:

1. Heparin administered I.V. causes activation of lipoprotein lipase, which tends to increase the relative migration rate of the fractions, especially the Beta lipoprotein.[24]
2. For effects of various drugs, refer to Young et al.[25]

Specimen Storage: For best separation of the various lipoproteins, fresh serum should be used. If testing cannot be performed immediately, the sample should be stored at 2 to 8°C no longer than 4 days. The specimen should never be stored frozen. Freezing may irreversibly alter the lipoprotein separation.[26] No additives or preservatives are necessary.

PROCEDURE

Materials Provided: The following materials are provided in the SPIFE Cholesterol Kits. Individual items are not available.

SPIFE Cholesterol Gels (10)
SPIFE Cholesterol Reagent (10 x 2.5 mL)
SPIFE Cholesterol Diluent (1 x 25 mL)
Citric Acid Destain (1 pkg)
REP Blotter C (10)
Electrode Blotter (20)
Applicator Blade Assembly - 20 Sample

Materials provided by Helena but not contained in the kit:

	Cat. No.
SPIFE 3000	1088
QuickScan Touch Plus	1640
Cholesterol Profile Control	3218
REP Prep	3100
SPIFE Gel Block Remover	1115
SPIFE Reagent Spreaders	3706
SPIFE Disposable Cups (deep well)	3360
SPIFE 2000/3000 20 -100 Disposable Cup Tray	3366
SPIFE Disposable Stainless Steel Electrodes	3388

STEP BY STEP METHOD

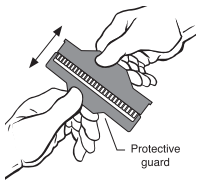
NOTE: If a SPIFE procedure requiring a stain has been run prior to running the cholesterol gels, the stainer unit must be cleaned/ washed before washing the cholesterol gel.

The SPIFE 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 electrophoresis, this wash cycle should be initiated at least seven (7) minutes prior to the end of the run. 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 process the gel after electrophoresis.

I. Preparation of Reagent

1. Reconstitute the SPIFE Cholesterol Reagent with 2.5 mL SPIFE Cholesterol Diluent. Mix well by inversion.

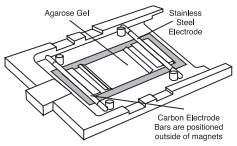
II. Sample Preparation



1. If testing 81 to 100 samples, remove five disposable Applicator Blades from the packaging. If testing fewer samples, remove the appropriate number of Applicator Blades from the packaging. Remove the protective guards from the blades by gently bending the protective piece back and forth until it breaks free.
2. Place the five Applicator Blades into the vertical slots in the Applicator Assembly identified as 2, A, 9, 13 and 16. Press on the end of each blade so that it slides to the back of the slot. If using fewer Applicator Blades, place them into any of the five slots noted above. **NOTE: The blade assembly will only fit into the slots one way; do not try to force the blade assembly into the slots.**
3. Slide cup strips into appropriate cup tray.
4. Pipette 75 to 80 µL of patient serum or control into Disposable Sample Cups. If testing less than 81 samples, pipette samples into the row of cups that corresponds with applicator placement. Cover the tray until ready to use.

III. Gel Preparation

1. Remove the gel from the protective packaging and discard overlay. Using a REP Blotter C, gently blot the entire gel using slight fingertip pressure on the blotter. Remove the blotter.
2. Dispense approximately 2 mL of REP Prep onto the left side of the electrophoresis chamber.
3. 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.
4. Thoroughly wash the electrodes with deionized water before and after each use. Wipe the carbon electrode with a lint-free tissue. The Disposable Electrode must be patted dry because of the rough surface. Ensure that the endcaps are screwed on tightly. The Disposable Electrode must be replaced after use on 50 gels. Unscrew the endcaps from the old electrode and screw them tightly onto the new electrode.



5. Place a carbon electrode on the outside ledge of the cathode gel block (left side of the gel) outside the magnetic posts.
6. Place a Disposable Stainless Steel Electrode on the outside ledge of the anode gel block (right side of the gel) outside the magnetic posts.
7. Place a glass rod on each inner gel block, inside the magnetic posts.
8. Place an Electrode Blotter directly above and below the cathode end of the gel. Slide the blotters under the ends of the carbon electrode so that they touch the gel block ends.
9. Press the **TEST SELECT/CONTINUE** button located on the Electrophoresis and Stainer sides of the instrument until **CHOLESTEROL** option appears on the display.

IV. Sample Application/Electrophoresis

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

Electrophoresis Unit

1) No Prompt				
Load Sample1	00:30	20°C	SPD6	
2) No Prompt				
Apply Sample1	1:00	20°C	SPD6	LOC1
3) No Prompt				
Electrophoresis 1	20:00	16°C	400 V	150mA
4) Remove blotter, (continue)				
Apply Reagent 1		30°C	8 cycles	
5) No Prompt				
Incubate 1	15:00	30°C		
6) No Prompt				
END OF TEST				

Stainer Unit

1) No Prompt			
Wash 1	5:00	REC = REV	VALVE = 2
2) Prompt			
Wash 2	5:00	REC = REV	VALVE = 7
3) No Prompt			
Dry 1	20:00	70°C	
4) No Prompt			
END OF TEST			

1. Place the Cup Tray with samples on the SPIFE 3000. Align the holes in the tray with the pins on the instrument.
2. Place a reconstituted vial of reagent in the center hole of the reagent bar, ensuring that the vial is pushed down as far as it can go. Close the chamber lid.
3. With **CHOLESTEROL** 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.
4. Open the chamber lid, remove and dispose of Electrode Blotters. Dispose of blades as biohazardous waste.
5. Close the chamber lid and press the **TEST SELECT/CONTINUE** button to pour, spread reagent and start the incubation timer.
6. At the end of the incubation, remove the gel from the chamber and place it on a blotter, agarose side up. Using a blade or straight edge, completely remove and discard the two gel blocks from the gel. The gel blocks interfere with washing.

V. Washing

1. Attach the gel to the 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.
2. Place the Gel Holder with the attached gel facing backwards into the stainer chamber.
3. With **CHOLESTEROL** 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 wash and dry the gel.
4. When the gel has completed the process, the instrument will beep. Remove the Gel Holder from the stainer and you can scan the bands.

Evaluation of Fractions

For quantitation of the lipoprotein cholesterol fractions, scan the gel, agarose side up, in the QuickScan Touch Plus on the Acid Violet setting. A silt size of 4 is recommended. Autoedit is used with this test.

Stability of End Product:

For best results, scan the SPIFE Cholesterol Gel within 5 minutes.

Calibration:

A calibration curve is not necessary as relative density of the fractions is the only parameter determined.

Quality Control: Quantitation of HDL Cholesterol values should be monitored using the Cholesterol Profile Control (Cat. No. 3218). This control verifies all phases of the procedure and should be used on each gel run. Refer to the package insert provided with the control for detailed information and assay values.

REFERENCE VALUES

Lipoprotein cholesterol values vary according to age and sex,[26] and wide variations among different geographical locations and races have been reported.[6] Therefore, it is essential that each laboratory establish its own expected range for its particular population. A total of 60 patients with normal total cholesterol (total cholesterol ≤ 200 mg/dL) were tested using the SPIFE Cholesterol system. These patients have not been differentiated by age, race or sex.

	Range ($\bar{x}$ ± 2 SD)
HDL (%)	10.7 - 37.7
Lp(a)-C%	0.0 - 10.3
VLDL (%)	0.0 - 33.6
LDL (%)	45.8 - 80.4

These values should only serve as guidelines. Each laboratory should establish its own range for age, sex and race.

RESULTS

The SPIFE Cholesterol system separates the major lipoprotein classes. The alpha band which migrates the farthest toward the anode corresponds to HDL. The next band, pre-beta, corresponds to VLDL. If a band appears between alpha and pre-beta, it is the Lp(a)-C band and should be added to the LDL quantitation when reporting the total LDL value.[27] It does not appear in every sample at measurable concentrations. The slowest moving beta band corresponds approximately to LDL. Chylomicrons, if present, remain at the origin.

Calculations

Helena densitometers will automatically calculate and print the relative percent and the absolute values for each band when the specimen total cholesterol is entered. Refer to the Operator's Manual provided with the instrument.

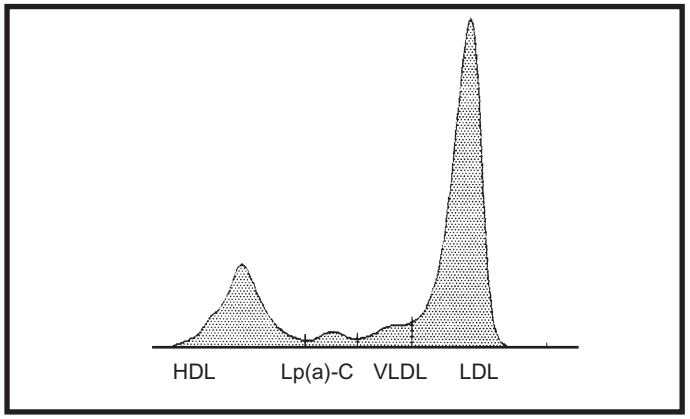


Figure 1: A scan of a SPIFE Cholesterol pattern.

LIMITATIONS

This method is intended for the separation and quantitation of lipoprotein classes. Refer to the SPECIMEN COLLECTION AND HANDLING section of this procedure for interfering factors.

The system is linear to 400 mg/dL total cholesterol, with sensitivity to 2.5 mg/dL per band. Patient sample quantitations which exceed the linearity of the system should be diluted with deionized water and retested.

Lp(a)-C below the threshold level of 2.5 mg/dL may not be seen using this method, even if Lp(a)-C is present in the sample. To quantitate patients who have an Lp(a)-C below 2.5 mg/dL it is recommended that an alternative method be used.

INTERPRETATION OF RESULTS

Treatment decisions in the NCEP guidelines are based primarily on LDL cholesterol levels.[10] The risk factors considered in the classification scheme are age (males equal to or older than 45 years and females equal to or older than 55), family history of premature CHD, smoking, hypertension, and diabetes. Treatment is appropriate when LDL cholesterol is at or above the following cut points: all patients at or above 160 mg/dL, with two or more risk factors a value above 130 mg/dL and with symptoms of CHD a value above 100 mg/dL.

HDL cholesterol is considered high risk at or below 35 mg/dL and counted as one of the risk factors in the classification scheme. An HDL cholesterol value above 60 mg/dL is considered protective and subtracts one from the total number of risk factors.

Treatment Decision Cut-Points[10]

Total Cholesterol		
Desirable Blood Cholesterol		< 200 mg/dL
Borderline-High Blood Cholesterol		200-239 mg/dL
High Blood Cholesterol		≥ 240 mg/dL
HDL Cholesterol		
Low HDL Cholesterol		< 40 mg/dL
Protective HDL Cholesterol		≥ 60 mg/dL
Triglycerides		
Desirable		< 150 mg/dL
Borderline		150-199 mg/dL
Elevated		200-499 mg/dL
Very elevated		≥ 500 mg/dL
LDL Cholesterol	Initiation	
Dietary Therapy	Level	LDL Goal
Without CHD and fewer than 2 risk factors	≥ 160 mg/dL	< 160 mg/dL
Without CHD and with 2 or more risk factors	≥ 130 mg/dL	< 130 mg/dL
With CHD	> 100 mg/dL	≤ 100 mg/dL
LDL Cholesterol	Initiation	
Drug Treatment	Level	LDL Goal
Without CHD and fewer than 2 risk factors	≥ 190 mg/dL	< 160 mg/dL
Without CHD and with 2 or more risk factors	≥ 160 mg/dL	< 130 mg/dL
With CHD	≥ 130 mg/dL	< 100 mg/dL

PERFORMANCE CHARACTERISTICS

PRECISION

Within Run

A patient sample was run 100 times on 1 gel of SPIFE Cholesterol.
N = 100

	HDL %	Lp(a)-C	VLDL %	LDL %
Mean	17.5	5.6	15.3	61.5
SD	0.9	0.2	0.5	0.9
CV	5.3%	4.3%	3.2%	1.5%

Run to Run

A patient sample was run 100 times on 6 gels of SPIFE Cholesterol.
N = 600

	HDL %	Lp(a)-C	VLDL %	LDL %
Mean	18.0	5.7	14.8	61.6
SD	1.1	0.5	1	1.4
CV	5.8%	7.8%	6.6%	2.3%

LINEARITY AND SENSITIVITY

Serial dilutions of an elevated cholesterol sample were made and tested by this system. The linearity study showed that the system is linear to 400 mg/dL total cholesterol and that the system is sensitive to 2.5 mg/dL per band.

CORRELATION STUDIES

A total of 131 patient samples, 72 normal (total cholesterol ≤ 200 mg/dL) and 59 abnormal (total cholesterol > 200 mg/dL), were run using SPIFE Cholesterol as the reference method. The following is the correlation data produced.
N = 131
Observations = 524
R = 0.996
Y = 1.026X - 0.623
X = SPIFE Cholesterol, 60 sample, acetic acid wash
Y = SPIFE Cholesterol, 100 sample, citric acid wash

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