SPIFE® LD Isoenzyme Procedure

Cat. No. 3335, 3336, 3337

INTENDED USE

The SPIFE LD Isoenzyme Procedure is intended for the qualitative and quantitative analysis of the lactate dehydrogenase isoenzymes in serum or plasma by agarose electrophoresis using the SPIFE 3000 systems.
For In Vitro Diagnostic use.
Rx Only

SUMMARY

Lactate dehydrogenase (LD) (EC 1.1.1.27) is an enzyme found in virtually all human tissues, with the liver, skeletal muscle, heart and kidney having the greatest concentrations. The wide distribution of LD in body tissues limits the usefulness of total LD determinations in diagnosis. Testing for the source of elevated LD activity may be indicated with isoenzyme assessment.[1]

Five isoenzymes of LD can be demonstrated in human serum. Each isoenzyme is designated by a number which is related to its electrophoretic mobility. The most anodic fraction is designated LD_1 and is found primarily in heart muscle. The most cathodic is LD_5 found primarily in liver and skeletal muscle. The others - LD_2, LD_3, and LD_4 are found in varying degrees along with LD_1 and LD_5 in all tissues.[1,4] Since LD_2 is found in highest concentration in normal human serum, the ratio LD_1/LD_2 is therefore less than one. Approximately 12-24 hours following myocardial infarction (MI), there is substantial elevation in LD_1 so that the LD_1/LD_2 ratio following MI will approach or even exceed 1, a phenomenon referred to as “flipped LD”. Peak activity is usually reached on day 3-4 and activity may remain elevated for as long as two weeks after infarction.[4] The LD “flip” can also be present in pernicious, hemolytic, acute sickle cell or megaloblastic anemias; renal necrosis or in cases of in-vitro or in-vivo hemolysis of any cause.[5]

An elevation of LD_5 can be seen in skeletal (muscle) injuries and degenerative diseases. It is also increased in many types of liver injuries such as cirrhosis, all types of hepatitis, and passive liver congestion.[5]

The mid-zone fractions (LD_2, LD_3, LD_4) may be elevated in cases of massive platelet destruction (pulmonary embolism) and in diseases involving the lymphatic system such as infectious mononucleosis, lymphomas and lymphocytic leukemias.[5]

The isoenzymes of LD have been determined by various methods.[7-11] Electrophoresis provides far more information than the other methods because it allows complete separation of all five isoenzymes with no risk of carryover. The support media used in electrophoresis includes cellulose acetate, agar, agarose and acrylamide gels.[1] The SPIFE LD system is a modification of that of Preston.[8]

PRINCIPLE

The isoenzymes of LD are separated according to their electrophoretic mobility on agarose. After separation, each isoenzyme is detected colorimetrically.

Using the SPIFE LD Isoenzyme System, a tetrazolium salt is reduced with the formation of a colored formazan dye.

$$\text{L-lactate} + \text{NAD} \xrightarrow[\text{Phenazine Methosulfate}]{\text{LD}} \text{Pyruvate} + \text{NADH}$$

$$\text{NADH} + \text{Tetrazolium Salts} \xrightarrow[\text{Phenazine Methosulfate}]{\text{LD}} \text{NAD} + \text{Formazan Dye}$$

REAGENTS

1. **SPIFE LD Isoenzyme Gel**
   Ingredients: Each gel contains agarose in a sodium barbital buffer, AMPD, aspartic acid, bicine and stabilizers. Sodium azide has been added as a preservative.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. The gel contains barbital which, in sufficient quantity, can be toxic. Refer to Sodium Azide Warning.
   Preparation for Use: The gels are ready for use as packaged.
   Storage and Stability: The gels should be stored 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 the gel blocks.
2. **LD Isoenzyme Reagent**
   Ingredients (after reconstitution):

NAD	10.0 mM
Lithium lactate	300.0 mM
NBT	11.1 mM
PMS	0.375 mM

   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST.
   Preparation for Use: Reconstitute each of two vials of reagent with 1.0 mL of LD Isoenzyme Diluent.
   Storage and Stability: The dry reagent should be stored at 2 to 6°C and is stable until the expiration date indicated on the vial. The reconstituted reagent is stable 48 hours at 2 to 6°C when stored in the dark. If exposed to the light, the color will change from yellow to green to blue. This does not affect the performance characteristics of the reagent.
   Signs of Deterioration: If the unreconstituted reagent is not a uniformly pale or light yellow, dry powder, it should not be used.
3. **LD Isoenzyme Diluent**
   Ingredients: The diluent is an AMP, bicine, barbital, aspartate buffer with sodium azide added as a preservative.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST. Refer to Sodium Azide Warning.
   Preparation for Use: The diluent is ready for use as packaged.
   Storage and Stability: The diluent should be stored at 2 to 6°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. DO NOT INGEST - IRRITANT.
   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 15-30°C. It is stable until the expiration date on the package.
   Signs of Deterioration: Discard if solution becomes cloudy.

Sodium Azide Warning

To prevent the formation of toxic vapors, sodium azide should not be mixed with acidic solutions. When discarding reagents containing sodium azide, 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.

INSTRUMENTS

A SPIFE 3000 must be used to electrophorese the gels. 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 instructions.

SPECIMEN COLLECTION AND HANDLING

Specimen: Serum is the specimen of choice. Plasma from blood specimens collected in heparin or EDTA may be used. Anticoagulants containing oxalate should not be used due to the inhibition of LD by oxalate.[11] Plasma samples should be well centrifuged to eliminate platelets which contain LD.[12]

Interfering Substances:

1. Hemolysis: Erythrocytes contain 100-150 times more LD than does serum. Hemolysis may contribute to error in assessment of $LD_{1,2}$ activity.[1-2,11]
2. Uremic sera: LD activity is reduced in uremic sera due to the presence of the inhibitors, urea and oxalate, and other unidentified substances. Urea affects LD_5 more than LD_1.[13]
3. Acetone and chloroform inactivate all isoenzymes of LD except LD_1.[14]
4. For the effect of various drugs on LD activity, refer to Young, et al.[15]

Storage and Stability: Serum should be tested as soon as possible after collection. Fresh serum is the specimen of choice because different storage conditions have varying effects on the isoenzymes.[11,14,16,17] No one storage temperature is optimum for all the isoenzymes. When storage is required, serum samples may be stored at 15 to 30°C or at 2 to 6°C for up to 48 hours. Storage at 2 to 6°C permits simultaneous storage of serum for both CK and LD isoenzyme studies.[11] Do not freeze the sample as LD_5 is very unstable at freezing temperatures.[11]

PROCEDURE

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

Sample Test Size	Cat. No.
60 sample	3335
40 sample	3336
20 sample	3337

SPIFE LD Isoenzyme Gels (10)
LD Isoenzyme Reagent (20 x 1.0 mL)
LD Isoenzyme Diluent (2 x 10 mL)
REP Blotter C (10)
Applicator Blade Assembly-20 Sample
Citric Acid Destain (1 pkg)

Materials provided by Helena but not contained in the kit:

	Cat. No.
SPIFE 3000	1088
QuickScan Touch Plus	1640
CK/LD Control	5134
REP Prep	3100
SPIFE Gel Block Remover	1115
SPIFE 3000/REP 3 Reagent Spreaders	3706
SPIFE 2000/3000 20-100 Dispo Cup Tray	3366
SPIFE Dispo Sample Cups (Deep Well)	3360
Chamber Cover	8JP34012

STEP-BY-STEP METHOD

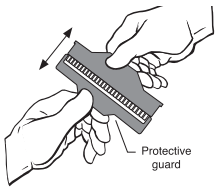
NOTE: If a SPIFE procedure requiring a stain has been run prior to running the LD gels, the stainer unit must be cleaned/washed before washing the gel.

The SPIFE 3000 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 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 Isoenzyme Reagent

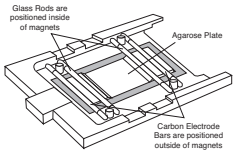
1. Reconstitute two vials of the LD Isoenzyme Reagent with 1.0 mL LD Isoenzyme Diluent each.
2. Mix well by inversion.

II. Sample Preparation



1. If testing 41-60 samples, remove three disposable Applicator Blade Assemblies from the packaging. If testing fewer samples, remove the appropriate number of Applicator Assemblies 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 three Applicator Blades into the vertical slots in the Applicator Assembly identified as 2, 9 and 16. If using fewer Applicator Blades, place them into any of the three 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 three Disposable Cup strips into rows 1, 3 and 5 of the cup tray.
4. Pipette 75-80 µL of patient serum or control into each cup. If testing less than 41 samples, pipette samples into the row of wells 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. Discard 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 gel backing, especially next to electrode posts, to remove excess REP Prep. Make sure no bubbles remain under the gel.
4. Clean and wipe the electrodes and the Reagent Spreaders (glass rods) with lint-free tissue.
5. Place a carbon electrode on the outer ledge of each gel block on the outside of the magnetic posts.
6. Place a Reagent Spreader (glass rod) on each inner gel block, inside the magnetic posts.
7. Press the **TEST SELECT/CONTINUE** button located on the Electrophoresis and Stainer sides of the instrument until **LD** 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 Sample 1	00:30	20°C	SPD6	
2) No Prompt Apply Sample 1	00:30	20°C	SPD6	LOC1
3) No Prompt Electrophoresis 1	6:00	10°C	600 Volt	
4) No Prompt Apply Reagent 1		45°C	4 cycles	
5) To Continue, (Continue) Incubate 1	20:00	45°C		
6) No Prompt END OF TEST				

Stainer Unit

1) No Prompt Destain 1	15:00	REC=REV	VALVE=2
2) No Prompt Wash 1	10:00	REC=REV	VALVE=7
3) No Prompt Dry 1	25:00	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 into each outer hole of the reagent bar, ensuring that the vials are pushed down as far as they can go. Close the chamber lid.
3. With **LD** 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, spread reagent, and beep.
4. Open the chamber lid, remove and dispose of blades as biohazardous waste.
5. Insert a Chamber Cover in the grooves of the chamber.
6. Close the chamber lid and press the **TEST SELECT/CONTINUE** button to start the incubation timer.

V. Washing

1. 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. Rinse the Chamber Cover before reuse.
2. 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.
3. Place the Gel Holder with the attached gel facing backwards into the stainer chamber.
4. With **LD** 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.
5. 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 of the LD Isoenzyme Bands

1. Qualitative evaluation: The SPIFE LD Isoenzyme Gel may be visually inspected for the bands.
2. Quantitative evaluation: Scan the SPIFE LD Isoenzyme Gel in the QuickScan Touch Plus on the Acid Violet setting using slit 5.

Stability of End Product

The LD gels should be scanned for quantitative results within two hours after drying. The gel should be protected from light in the interim.

Calibration

A calibration curve is not necessary because relative intensity of the bands is the only parameter determined.

Quality Control

The CK/LD Isoenzyme Control (Cat. No. 5134) 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 proper location of the isoenzyme bands and may also be quantitated to verify the accuracy of quantitations. Refer to the package insert provided with the control for assay values. Additional controls may be required for federal, state or local regulations.

REFERENCE VALUES

Reference range studies including fifty-three healthy men and women between the ages of 20 and 60 years old were performed by Helena Laboratories. The following results were obtained:

LD_1	=	17.4 - 30.0%
LD_2	=	29.6 - 40.6%
LD_3	=	20.6 - 27.8%
LD_4	=	5.1 - 10.1%
LD_5	=	3.6 - 15.4%
LD_1/LD_2	=	0.5 - 0.9

These values should only serve as guidelines. Each laboratory should establish its own expected value range with this procedure.

RESULTS

Following electrophoresis, five zones of LD activity can be demonstrated. The most anodic zone (LD_1) migrates with a mobility similar to $alpha_1$ globulin. The most cathodic zone (LD_5) travels with the gamma globulin and the remaining three zones have intermediate mobilities. The LD activity in normal serum reflects the breakdown of numerous cells and all 5 components can be seen. LD_2 predominates, followed by LD_1. LD_3 is present in moderate amounts while LD_4 and LD_5 usually occur only in minor amounts.

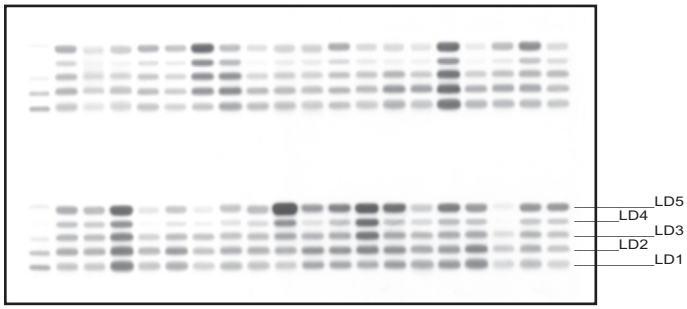


Figure 1: SPIFE LD Gel showing the relative position of the LD isoenzyme bands.

Calculation of the Unknown The QuickScan Touch Plus densitometer will automatically calculate and print the relative percent and the absolute values for each band. Refer to the Operator's Manual provided with the instrument.

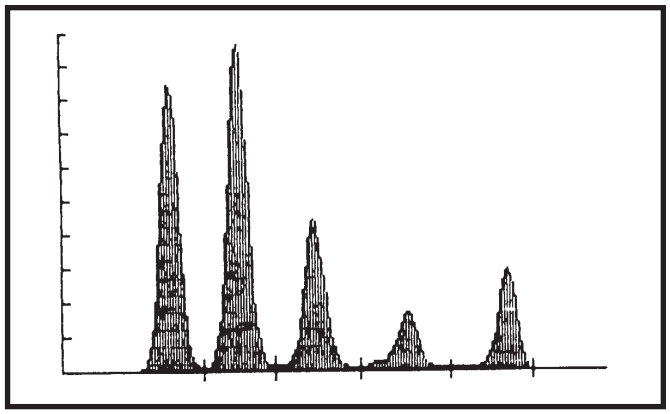


Figure 2: A representative scan of a SPIFE LD pattern.

LIMITATIONS

The SPIFE LD Isoenzyme Reagent is linear to a total LD of 1000 U/L. Samples with values greater than this should be diluted with deionized water. Results from sensitivity studies showed that the SPIFE LD Reagent is sensitive to 3 U/L.

NOTE: The SPIFE LD method is not designed to identify tumor markers.

Interfering factors: Refer to SPECIMEN COLLECTION AND HANDLING

Further Testing Required:

1. Total LD activity may be determined. Conflicting reports exist about the true value of total serum enzyme levels as compared to the severity of a disease.[1,4,22]
2. In diagnosing myocardial infarction, CK isoenzyme studies should be performed.[1,4]
3. Haptoglobin studies may be performed to rule out hemolysis as a cause of elevated LD_1 and LD_2.

INTERPRETATION OF RESULTS

1. LD_2 is the LD isoenzyme present in the largest amount in normal serum.[1-4,11]
2. LD_1 is elevated and may be greater than LD_2 in:
   a. Myocardial infarction.[1-4,11]
   b. Duchenne's muscular dystrophy presents a pattern like MI but clinical symptoms help in easily differentiating the two diseases.[18-19]
   c. Hemolysis (including Hemolytic anemias). Hemolytic anemias should be strongly considered whenever total serum LD reaches levels greater than 5 times normal, and the isoenzymes show an increased LD_1 and LD_2. Total LD is much higher in hemolytic anemia than in MI unless MI is accompanied by severe shock. Pernicious anemia (PA) in relapse gives an LD pattern like hemolysis. Some of the highest total serum LD values are found in PA.[2,14]
   d. Renal infarct.[2,11]

3. LD_3 is elevated in pulmonary infarctions.[6,11,20]
4. LD_4 elevation has not been associated with any particular pathology.
5. LD_5 is elevated in hepatic and muscular damage and diseases of the skin.[1]
6. Isomorphic patterns:
   When total LD is markedly elevated but all the isoenzymes are of normal percentages, the phenomenon is referred to as an isomorphic pattern. Widely divergent groups of clinical diagnoses have shown this type of pattern and include cardiorespiratory diseases, malignancy, fracture, diseases of the central nervous system, infection/inflammation, hepatic cirrhosis and/or alcoholism, trauma without fracture, infectious mononucleosis, hypothyroidism, uremia, necrosis, pseudomononucleosis, viremia and intestinal obstruction. (See Limitations Note)
7. CK and LD values following open heart surgery:
   CK and LD isoenzymes are less specific following open heart surgery than they are in most diagnostic situations. The CK-MB will be elevated due to myocardial damage resulting from the operative procedure as well as trauma to the heart from manipulation and cannulation. The LD_1/LD_2 may be elevated secondary to hemolysis from extra corporeal circulation.

PERFORMANCE CHARACTERISTICS

SPIFE 3000

PRECISION

Within Run studies were run using a patient sample run in replicate on one gel. N = 60

Fraction	Mean	SD	CV%
LD_1	26.8	0.5	1.7
LD_2	32 3	0.5	1.4
LD_3	22.7	0.3	1.5
LD_4	8.5	0.3	4.0
LD_5	9.8	0.4	4.6

Between Run studies were done using a patient sample run in replicate on five gels. N = 300

Fraction	Mean	SD	CV%
LD_1	27.1	0.6	2.4
LD_2	31.5	0.9	2.8
LD_3	23.0	0.4	1.9
LD_4	8.6	0.6	6.5
LD_5	9.8	0.6	5.8

CORRELATION STUDIES

132 patient specimens were tested on the SPIFE LD method and another commercially available product.

N	= 132
Slope	= 0.999
Intercept	= 0.151
R	= 0.980
Y	= 0.999X + 0.151
X	= TITAN GEL LD
Y	= SPIFE LD

LINEARITY

The system has been validated to be linear to 1000 IU/L total LD.

SENSITIVITY

Results from validation studies show that the system is sensitive to 3 IU/L.

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