REP® LD-16
Isoenzyme Procedure

Cat. No. 3076

The REP LD-16 Isoenzyme Procedure is intended for the qualitative and quantitative analysis of the lactate dehydrogenase isoenzymes in serum or plasma by agarose electrophoresis.

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 fastest moving fraction (most anodic) is designated LD1 and is found primarily in heart muscle. The slowest moving (most cathodic) is LD5 found primarily in liver and skeletal muscle. The others - LD2, LD3, and LD4 are found in varying degrees along with LD1 and LD5 in all tissues.[1, 4]

The most important use of LD isoenzymes is in the diagnosis of myocardial damage. LD2 is found in highest concentration in normal human serum. The ratio LD1/LD2 is therefore less than one. Following myocardial infarction (MI), there is substantial elevation in LD1 so that the LD1/LD2 ratio following MI will approach or even exceed 1, a phenomenon referred to as "flipped LD." The LD level begins to rise approximately 12-24 hours following myocardial infarction, frequently reaching levels two to three times (or greater) the upper limit of normal. Peak activity is usually reached on day 3-4 and activity may remain elevated for as long as two weeks after infarction.[4]

The most definitive testing in the diagnosis of MI is accomplished by performing creatine kinase (CK) isoenzyme studies in conjunction with LD isoenzyme studies.[1-5] The specificity and sensitivity achieved with these two tests has eliminated the necessity for additional enzyme studies in accurately diagnosing MI. Studies have shown that CK and LD isoenzyme analyses, in conjunction with the proper clinical setting and electrocardiogram results, are virtually 100% accurate in properly diagnosing myocardial infarction.[4, 6]

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 REP 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 fluorometrically. The REP LD-16 Isoenzyme Reagent utilizes the following reaction:[1]

REAGENTS

1. **REP LD-16 Isoenzyme Gel**
   Ingredients: Each gel contains agarose in an AMP/MOPSO buffer. Sodium azide has been added as a preservative.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. 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 gel blocks.
2. **REP LD-16 Isoenzyme Reagent**
   Ingredients: Lithium lactate ....937 mM
   NAD..14 mM
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST.
   Preparation for Use: Reconstitute each vial of reagent with 1.0 mL of REP 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. Reconstituted reagent is stable for 24 hours at 4°C.
   Signs of Deterioration: If the unreconstituted reagent is not a uniformly white or slightly off-white dry powder, it should not be used.
3. **REP LD Isoenzyme Diluent**
   Ingredients: The diluent is an AMP and Hepes buffer solution 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.

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 Rapid ElectroPhoresis Analyzer (REP or REP 3) must be used to electrophorese, apply reagent and scan the gels. Refer to the 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.[12] Plasma samples should be well centrifuged to eliminate platelets which contain LD.[13]

Collection of Specimen: Proper timing of specimen collection is extremely critical for accurate interpretation of LD isoenzyme assessment in the diagnosis of myocardial infarction. A minimum of three specimens should be collected. The first samples should be collected immediately upon admission of the patient to the hospital. The second specimen should be collected 6-13 hours later and the third specimen collected 24-37 hours after admission.[2-4]

Interfering Substances:

1. Hemolysis: Erythrocytes contain 100-150 times more LD than does serum. Hemolysis may contribute to error in assessment of LD1, 2 activity.[1-2, 12]
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 LD5 more than LD1.[14]
3. Acetone and chloroform inactivate all isoenzymes of LD except LD1.[15]
4. For the effect of various drugs on LD activity, refer to Young, et al.[16]

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.[12,15,17,18] 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.[12] Do not freeze the sample as LD5 is very unstable at freezing temperatures.[12]

PROCEDURE

Materials Provided: The following materials are provided in the REP LD-16 Isoenzyme Kit. Individual items are not available separately.

REP LD-16 Isoenzyme Gels (10)
REP LD-16 Isoenzyme Reagent (10 x 1.0 mL)
REP LD Isoenzyme Diluent (1 x 15 mL)
REP Blotter A (10)
REP Sample Cups (160 cups)

Materials provided by Helena but not contained in the kit:

	Cat. No.
REP CK/LD Isoenzyme Control	3073
REP Prep	3100
SUREprep	1574
REP Prepper	1359

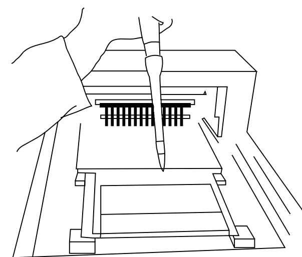
STEP-BY-STEP METHOD

A. Preparation of Isoenzyme Reagent

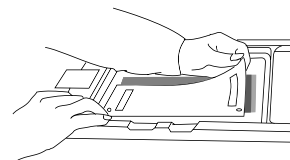
1. Reconstitute the REP LD-16 Isoenzyme Reagent with 1.0 mL REP LD Isoenzyme Diluent.
2. Mix well by inversion.

B. Sample Application

1. Place 8 sample cups into wells 4, 5, 6, 7, 8, 9, 10 and 11; and 8 cups into wells 19, 20, 21, 22, 23, 24, 25 and 26; (color coded with a green stripe). Place 50-75 µL of sample in each sample cup. Place REP Blotter A on sample tray in area adjacent to sample cups. Place approximately 4 mL of SUREprep into outside washwell of sample tray. Place approximately 4 mL of water into inside washwell of sample tray.

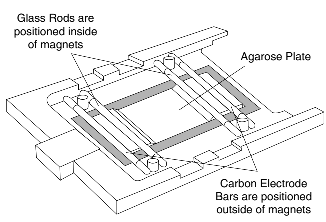


2. Dispense approximately 1 mL of REP Prep into left side of chamber.
3. Remove the gel from the protective packaging and discard overlay. Use a REP Prepper or compressed air to blow excess moisture from the wells and the gel surface. Inspect the scanning area of the gel for surface artifacts.
4. Place left edge of gel over REP Prep aligning the round hole on the left pin. 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 paper towel or absorbent paper to wipe around edges of gel, especially next to electrode posts, to remove excess REP Prep.



Make sure that the gel lays flat and that no bubbles remain under the gel.

5. Clean and wipe the electrodes with lint-free tissue.
6. Place a carbon electrode on the outer ledge of each gel block on the outside of the magnetic posts. Place a glass rod on the inner ledge of each gel block on the inside of the magnetic posts.



7. Place the open vial of reconstituted reagent firmly in the center vial holder (color coded with a green stripe).
8. Slide the lid into place until it snaps.
9. Using the instructions provided in the appropriate Operator's Manual, set up parameters on the screen as follows:

REP

Sample Location (Row) .... AB
Sample Application Time .... 1 sec
Application Volume .... 1 µL
Sample Absorption Time .... 1:00 mm:ss
Needle Wash Cycles 2
Needle Blot Time .... 1 sec
Electrophoresis Time .... 5:00 mm:ss
Electrophoresis Voltage .... 1000 volts
Electrophoresis Current .... 0 mA
Electrophoresis Temp. .... 10°C
Air Dry Time .... mm:ss
Reagent Pour Time .... 1 sec
Reagent Spread Cycles 4
Incubation Time .... 4:30 mm:ss
Incubation Temperature .... 45°C
Dry Time .... 5:00 mm:ss
Dry Temperature .... 54°C
Standby Temperature .... 16°C

Depress the F1 key, the REP unit will automatically apply samples, electrophorese, apply reagent, incubate and dry the agarose gel.

After the run is completed, 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 gel blocks from both ends of the gel.

REP 3

Sample Application Volume................................1.0 µL
Sample Application Row A.........66.50 mm from front pin
Sample Application Row B.......117.00 mm from front pin
Sample Absorption Time.........................00:40 min:sec
Electrophoresis Voltage ....1000 volts
Electrophoresis Current Limit ....300 mA
Electrophoresis Temperature ....10°C
Electrophoresis Time ....05:00 min:sec
Air Dry Time...00:00 min:sec
Reagent Spread Cycles...4
Reagent Absorption Time ....00:02 min:sec
Center Electrode State...None
Incubation Temperature.......................................45°C
Incubation Time......................................04:30 min:sec
Dry Temperature ....54°C
Dry Time ....05:00 min:sec

Touch the "Start Run" area on the touch screen. The REP 3 will automatically apply samples, electrophorese, apply reagent, incubate, dry and scan the gel.

C. Evaluation of the LD Isoenzyme Bands

1. Qualitative evaluation: The REP LD-16 Isoenzyme Gel may be visually inspected under a UV lamp for the presence of the bands.
2. Quantitative evaluation: Scan the REP LD-16 Isoenzyme Gel in the REP using the fluorescence mode. Place the agarose side of the gel down toward the detector. The REP 3 will automatically scan the gel.

Stability of End Product

The LD gels should be scanned for quantitative results within one hour 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 REP CK/LD Isoenzyme Control (Cat. No. 3073) 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.

REFERENCE VALUES

REP

Reference range studies including both men and women were performed by Helena Laboratories. The following results were obtained:

LD1	=	14.8 - 25.4%
LD2	=	31.7 - 41.4%
LD3	=	18.1 - 25.9%
LD4	=	7.2 - 13.6%
LD5	=	5.3 - 16.5%

REP 3

Reference range studies were performed by Helena Laboratories on 60 samples from healthy men and women. The results were as follows:

LD1	=	15.8 - 29.1%
LD2	=	32.6 - 42.0%
LD3	=	14.5 - 23.0%
LD4	=	7.4 - 13.8%
LD5	=	6.6 - 19.2%

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 fastest zone (LD1) migrates with a mobility similar to alpha1 globulin. The slowest zone (LD5) 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. LD2 predominates, followed by LD1. LD3 is present in moderate amounts while LD4 and LD5 usually occur only in minor amounts.

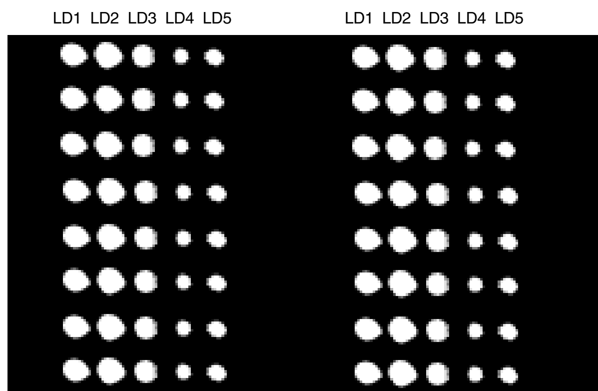


Figure 1: REP LD-16 Gel showing the relative position of the LD isoenzyme bands.

Calculation of the Unknown

The Helena REP 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.

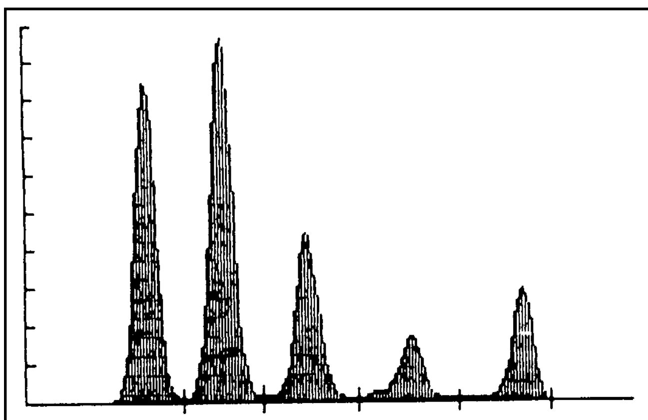


Figure 2: A representative scan of a REP LD pattern.

LIMITATIONS

The REP LD-16 Isoenzyme Reagent is linear to a total of 350 U/L per band or a total LD of 900 U/L. Samples with values greater than this should be diluted with deionized water. Total LD activity was determined using a U.V. kinetic lactate to pyruvate method at 30°C which has a normal range of 52-149 U/L. Results from sensitivity studies showed that the REP LD Reagent is sensitive to 2 U/L.

Note: The REP LD-16 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, 25]
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 LD1 and LD2.

PERFORMANCE CHARACTERISTICS

REP

PRECISION

Within Run: Reproducibility of the system was established using a patient sample run 16 times on the REP LD Isoenzyme Gel. The following results were obtained: N = 16

Fraction	Mean%	SD	CV%
LD1	29.9	0.5	1.7
LD2	35.9	0.8	2.2
LD3	16.7	0.5	3.0
LD4	6.5	0.4	3.8
LD5	11.0	0.5	4.9

Between Run: A control was run sixteen times on each of eleven (11) gels. N = 176

Fraction	Mean%	SD	CV%
LD1	29.3	0.8	2.8
LD2	35.7	0.8	2.2
LD3	17.4	0.9	5.2
LD4	7.0	0.6	8.0
LD5	10.7	0.7	6.4

CORRELATION

The Helena REP LD-16 method was compared to the REP LD-30 product with the following correlation:

N	= 30	Y =	0.997X + 0.048
Slope	= 0.997	X =	REP LD-16
Intercept	= 0.048	Y =	REP LD-30
r	= 0.993		

REP 3

PRECISION

Within Run studies were run using a control run in replicate on one gel. N = 16

Fraction	Mean%	SD	CV%
LD1	34.8	0.3	1.0
LD2	34.8	0.4	1.1
LD3	13.6	0.5	3.4
LD4	7.7	0.4	4.9
LD5	9.1	0.6	6.5

Between Run studies were done using a control run in replicate on eight (8) gels. N = 128

Fraction	Mean%	SD	CV%
LD1	34.3	0.8	2.4
LD2	34.3	0.9	2.5
LD3	13.9	0.5	3.7
LD4	7.9	1.0	12.6
LD5	9.6	0.9	9.2

LINEARITY

The system has been validated to be linear to 900 U/L total LD using a UV kinetic method at 37°C.

SENSITIVITY

Results from validation studies show that the system is sensitive to 2 U/L.

CORRELATION

Fifty (50) specimens, including 20 normals and 30 abnormals, were tested using both the REP and REP 3.

N	= 50	Y =	0.930X + 1.403
Slope	= 0.930	X =	LD-30 gels run on REP System
Intercept	= 1.403		
r	= 0.996	Y =	LD-16 gels run on REP 3 System

INTERPRETATION OF RESULTS

1. LD2 is the LD isoenzyme present in the largest amount in normal serum.[1-4, 12]
2. LD1 is elevated and may be greater than LD2 in:
   a. Myocardial infarction.[1-4, 12]
   b. Duchenne's muscular dystrophy presents a pattern like MI but clinical symptoms help in easily differentiating the two diseases.[19-20]
   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 LD1 and LD2. 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, 15]
   d. Renal infarct.[2, 12]
3. LD3 is elevated in pulmonary infarctions.[7, 12, 21]
4. LD4 elevation has not been associated with any particular pathology.
5. LD5 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.[1, 2, 22] (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 LD1/LD2 may be elevated secondary to hemolysis from extra corporeal circulation. Infarct patients have higher levels of CK-MB activity, but the wide range of isoenzyme activity seen in non-MI patients overlaps that noted in patients with MI. This makes complete discrimination impossible. Despite this difficulty, accuracy in diagnosing MI can be increased by analyzing serial determinations of CK-MB in the post-operative period. Perioperative infarct patients will usually have a progressive rise in CK-MB levels, while non-MI patients exhibit a more precipitous post-operative decrease in that fraction.[22-24]

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REP® LD-16 Isoenzyme System

REP LD-16 Isoenzyme Kit	Cat. No. 3076
REP LD-16 Isoenzyme Gels (10)	
REP LD-16 Isoenzyme Reagent (10 x 1.0 mL)	
REP LD Isoenzyme Diluent (1 x 15 mL)	
REP Blotter A (10)	
REP Sample Cups (160 cups)	

Other Supplies and Equipment

The following items, needed for performance of the REP LD-16 Isoenzyme Procedure, must be ordered individually.

	Cat. No.
REP (Rapid ElectroPhoresis) Analyzer	1352
REP 3	3700
REP CK/LD Isoenzyme Control (5 x 2.0 mL)	3073
REP Prep	3100
SUREprep	1574
REP Prepper	1359

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Helena Laboratories
Beaumont, Texas

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