

REP® Alkaline Phosphatase-15 Isoenzyme Procedure

Helena  Laboratories

Cat. No. 3200

The procedure is intended for the qualitative and/or semi-quantitative determination of serum alkaline phosphatase isoenzymes using specimen pretreatment with neuraminidase and agarose electrophoresis on the REP system.

SUMMARY

Alkaline phosphatase (ALP) (EC 3.1.3.1.) is an enzyme which catalyzes the hydrolysis of phosphate esters at an alkaline pH. The greatest concentrations of ALP are found in bone, liver, intestine, and the placenta. However, practically every body tissue contains at least a small amount of ALP. Because of this wide distribution, limited information can be obtained from a total ALP assay. Fortunately each source of ALP produces one predominant isoenzyme and the tissue source of elevated ALP in serum can be determined by identifying the isoenzyme. The isoenzymes of ALP differ in their physiochemical and electrophoretic properties and, by taking advantage of these differences, the individual isoenzymes can be identified.¹ In addition to the liver, bone, intestinal and macrohepatic isoenzymes, other ALP isoenzymes have been identified in serum. These include placental, Regan, Nagao, PA, and renal isoenzymes. The presence of these isoenzymes may interfere with the identification and quantitation of bone and liver by electrophoretic methods. A number of laboratory procedures have been used for the routine evaluation of the ALP isoenzymes. These include heat inactivation², inhibition with amino acids³⁻⁵, urea denaturation³⁻⁴, and electrophoresis on agarose⁶, paper⁷, starch gel^{8, 14, 15}, polyacrylamide gel^{9, 16} and cellulose acetate¹⁰⁻¹².

The REP ALP method offers several advantages over all existing methods in that macrohepatic, liver, bone and intestine are all clearly separated.

PRINCIPLE

The REP ALP Isoenzyme procedure is a high resolution method, and the isoenzyme migrations differ from those seen in conventional isoenzyme electrophoretic methods.

Certain specific neuraminidases remove sialic acid from enzymes, reducing the net negative charge, thus affecting their anodal electrophoretic mobility.¹³ Since bone alkaline phosphatase contains more sialic acid than the liver isoenzyme, the neuraminidase causes a greater reduction in mobility of the bone enzyme than the liver isoenzyme.¹³ Taking advantage of this, results in greater separation of these two isoenzymes. The macrohepatic alkaline phosphatase isoenzyme is also affected by neuraminidase so that it electrophoreses with the bone fraction when non high resolution techniques are used.

The use of a detergent in the agarose allows the separation of the bone and macrohepatic alkaline phosphatase bands causing the latter band to move slower. The presence of the intestinal isoenzyme does not interfere with electrophoretic patterns since its mobility is unaffected by neuraminidase.^{13, 15} Combining sample pretreatment and high resolution techniques allows the system to separate all four ALP isoenzymes (liver, bone, macrohepatic and intestine).

The data generated can be used as a clinical tool in the diagnosis and treatment of liver, bone, parathyroid and intestinal disorders. This high resolution system may separate 3 intestinal fractions, but the clinical significance of these has not been determined. The enzyme activity is developed using BCIP as the substrate and AMP as the phosphate acceptor.

REAGENTS

1. REP ALP Diluent

Ingredients: The concentration of the reactive ingredients is as follows:

2-Amino-2-Methyl,1-Propanol	2.0 M
5-Bromo-4-Chloro-3-Indolyl Phosphate-p-Toluidine salt	1.7 mM
Magnesium Sulfate	0.85 mM
Sodium Azide	0.1%

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 on the bottle.

Signs of Deterioration: The diluent should be destroyed if it becomes milky white or shows signs of contamination.

2. REP ALP Reagent

Ingredients: NBT (nitro blue tetrazolium) - 1.83 mM

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

Preparation for Use: Reconstitute each vial with 3 mL of REP ALP Diluent, and vortex well.

Storage and Stability: The dry powder should be stored at 2 to 6°C and is stable until the expiration date on the bottle. The reconstituted reagent and chromogen should be used within 30 minutes.

Signs of Deterioration: The powder should be a dry, light yellow color.

3. REP ALP-15 Gel

Ingredients: Each gel contains agarose in a tris-barbital-sodium barbital buffer. 0.15% sodium azide and other preservatives have been added.

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

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.

4. REP ALP Separation Enhancer

Ingredients: Neuraminidase from *Vibrio cholerae* (E.C. 3.2.1.18) and preservatives.

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

Preparation for Use: The product is ready for use as packaged.

Storage and Stability: Store at 2 to 6°C and is stable until the expiration date on the vial.

Signs of Deterioration: A normal isoenzyme pattern should separate into 2 bands if enzyme is functioning properly.

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 the REP 3) must be used to electrophorese, stain and scan the gel. Visual inspection for the presence of isoenzyme bands, indicating disease conditions, is sufficient. Refer to the appropriate Operators Manual for detailed operating instructions.

NOTE: A REP or REP 3 modified electrophoresis chamber lid must be used when electrophoresing this gel.

SPECIMEN COLLECTION AND HANDLING

Specimen: Serum is the specimen of choice. Anticoagulants containing oxalate, citrate or EDTA cannot be used because these substances inhibit the alkaline phosphatase activity.¹⁷ Total alkaline phosphatase activity should be determined.

Patient Preparation: The patient should be fasting. Patients who have B or O blood group and are secretors may have an elevated ALP about two hours after a fatty meal.^{6, 12, 17, 22, 23}

Interfering Substances:

1. High concentrations of phosphate, oxalate, citrate and cyanide will inhibit ALP activity.^{17, 22}
2. Excess glycine may inhibit ALP activity by complexing Mg⁺⁺.¹⁷
3. EDTA inhibits some of the isoenzymes of ALP. Do not use as an anticoagulant.¹⁷
4. Several drugs cause an enzymatic imbalance which may change the ALP level.^{17, 18}

Storage and Stability: It is preferable to refrigerate the blood specimen immediately after collection. Specimens should be separated from the red blood cells as soon as possible. It is strongly recommended that fresh serum samples be used. If storage is necessary, the serum should be stored frozen (-20°C) for no more than 24 hours.^{19, 20, 22}

PROCEDURE

Materials Provided: The following materials are provided in the REP Alkaline Phosphatase Kit.

- REP ALP-15 Gels (10)
- REP Alkaline Phosphatase Separation Enhancer (1.5 mL)
- REP ALP Reagent (10 x 45 mg)
- REP ALP Diluent (35 mL)
- REP Blotter A (10)
- REP Sample Cups (150)

Materials provided but not contained in the kit:

- | | |
|--|------|
| GEL Alkaline Phosphatase Control | 5104 |
| REP Prepper | 1359 |
| SUREprep | 1574 |
| REP Prep | 3100 |
| Modified Chamber Cover (supplied with REP) | |

Materials needed but not provided:

- Specimen sample cups - test tubes
- 10% Acetic Acid Solution - Destain solution

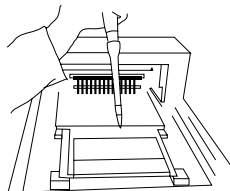
STEP-BY-STEP

A. Sample Pretreatment

1. Prepare each sample and control by mixing 10 µL of Separation Enhancer with 50 µL of sample in small test tube. Since enzymes degrade rapidly, use within 10 minutes.

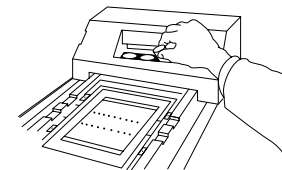
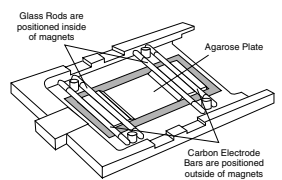
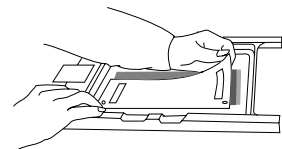
B. Sample Application

1. Place 15 sample cups into the sample tray. Place 50 µL of sample into 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 2 mL of REP Prep onto left side of REP chamber.
3. Remove the gel from the protective packaging and discard overlay. Use the REP Prepper or compressed air to blow excessive moisture from sample wells.
4. Place the left edge of the 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 a paper towel or absorbent paper to wipe around the edges of the gel, especially next to magnetic 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 a 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 in the center vial holder, (color coded with a blue stripe).
8. Slide the modified 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] A
Sample Application Time 1 sec
Sample Application Volume 1.0 µL
Sample Absorption Time 00:60 sec
Needle Wash Cycles 2
Needle Blot Time 1 sec
Electrophoresis Time 30:00 min:sec
Electrophoresis Voltage 430 volts
Electrophoresis Current 0 mA
Electrophoresis Temperature 12°C
Air Dry Time 0:00 min:sec
Reagent Pour Time 1 sec
Reagent Spread Cycles 10
Incubation Time 25:00 min:sec
Incubation Temperature 45°C
Dry Time 2:00 min:sec
Dry Temperature 54°C
Standby Temperature 16°C

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

REP 3

Sample Application Volume 1.0 µL
Sample Application Row A 66.00 mm from front pin
Sample Application Row B 117.00 mm from front pin
Sample Absorption Time 01:00 min:sec
Electrophoresis Voltage 430 volts
Electrophoresis Current Limit 90 mA
Electrophoresis Temperature 12°C
Electrophoresis Time 30:00 min:sec
Air Dry Time 00:00 min:sec
Reagent Spread Cycles 10

Reagent Absorption Time 00:00 min:sec
 Center Electrode State None
 Incubation Temperature 45°C
 Incubation Time 25:00 min:sec
 Dry Temperature 54°C
 Dry Time 02:00 min:sec
 Touch the "Start Run" area on the touch screen. The REP 3 will automatically apply samples, electrophorese, apply reagent, incubate and dry the gel.

10. At the end of the electrophoresis and drying period, remove the gel from the chamber and place it on a blotter, agarose side up. Using a blade or straight edge, remove the two gel blocks from the gel completely and discard the gel blocks.
11. Destain the gel in two (2) consecutive washes of destain solution. Use a vigorous, alternately rocking and swirling technique. Allow the gel to remain in each wash for 30 seconds. The gel background should be completely clear.
12. Dip the gel into a final water wash for a few seconds. Swirl or agitate the water during this step. Remove the gel from the water, and tap the gel to remove the excess water.
13. Dry the destained gel in the REP at 54°C for 3 minutes.

Evaluation of the Alkaline Phosphatase Isoenzyme Bands

Gels should be visually evaluated for band positions using a bone/liver control. Scan the dried ALP gels by placing the gel in the densitometer agarose side toward the light source/detector and with the wavelength at 595 nm. Since the GEL Alkaline Phosphatase Isoenzyme Control is quantitated, an approximate value for the patient sample can be derived by comparison to the control. The results can be reported as greater than or less than the control values.

Stability of End Product: Gels should be scanned and/or interpreted within 2 hours.

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

Quality Control: The GEL Alkaline Phosphatase Isoenzyme Control (Cat. No. 5104) verifies all phases of the procedure and should be used on each gel run. The control may be used as a marker for the proper location of the bands or it may be quantitated to verify the accuracy of quantitations in the procedure. Refer to the package insert provided with the control for assay values.

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 densitometer.

REFERENCE VALUES

Interpretation of isoenzyme patterns should not be attempted without knowledge of the total ALP level in the patient's serum. Serum from normal individuals may contain small amounts of liver, bone and intestinal ALP.^{10, 12, 21} ALP levels are age and sex dependent.²²⁻²⁴

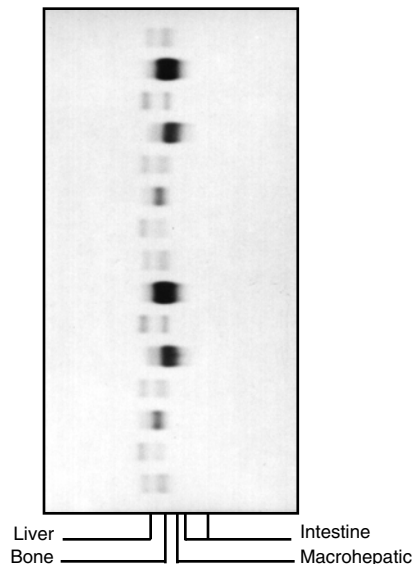
REP

Forty-eight samples were obtained from supposedly normal, non-fasting adults and were used to derive an expected range with the following results:

Liver	14.7 - 87.5%
Bone	12.5 - 85.3%
Intestine	0.0 - 6.4%

These values should only serve as guidelines. Each laboratory should establish its own range.

Pregnant women may show a placental band. The macrohepatic band seen in neoplasms, and referred to as fast liver, should be interpreted as an alert to a disease state regardless of the total ALP level. The performance of Nagao, Regan and PA with this system are not known at this time. Abnormal bands have been reported in patients with normal total alkaline phosphatase levels.



RESULTS

The liver band migrates the most anodic of all the bands. The liver band on patients with a high total will migrate more anodally than that on a normal level patient. The liver band is followed by a band in the bone position and then the macrohepatic (fast liver) band. In the presence of a high concentration of bone activity, the bone will migrate slower than that of a normal patient. With liver running fast and bone running slow, there is greater separation of the two bands. Three minor intestinal bands are occasionally seen, particularly on non fasting samples. All 3 of the intestinal bands migrate cathodic to the macrohepatic band. The intestinal bands are sharp and narrow, as is the macrohepatic band.

A control should be run with each gel to use as a band marker. Each unknown specimen should be compared to the control for band migration and approximate value of each isoenzyme.

INTERPRETATION OF RESULTS

LIVER ISOENZYME: Liver is the isoenzyme most frequently elevated when total ALP levels are elevated.^{10, 12} The liver ALP increases in the blood early in liver disease before most other liver function tests show abnormalities. The wide group of conditions leading to increased liver ALP include acute hepatitis, cirrhosis, fatty liver, drug induced liver disease, obstruction of biliary flow by carcinoma at the head of the pancreas, bile duct stricture, primary biliary cirrhosis, and metastatic carcinoma of the liver.²²

MACROHEPATIC ISOENZYME:¹¹ Macrohepatic ALP has been isolated in cases of metastatic carcinoma to the liver and has been suggested as a diagnostic tool in identifying such cases. It has also been isolated in patients with viral hepatitis, alcoholic cirrhosis and other liver diseases. Data generated in a study by Viot and his associates¹¹ suggest that hepatic ALP is highly correlated with the presence of liver metastases and that the presence of this isoenzyme could be predictive of the appearance of liver metastases. Viot also reports that macrohepatic ALP is seen occasionally in patients free of any disease state.¹¹

BONE ISOENZYME: Elevated as a result of increased osteoblastic activity. This isoenzyme is normally elevated in growing children and adults over the age of fifty. The highest total ALP values have been attributed to an increased bone isoenzyme level due to Paget's disease or renal rickets.²⁵ An abnormally high bone isoenzyme level may also be indicative of bone cancer, osteomalacia or coeliac sprue.²² A decreased bone ALP in children may be attributed to cretinism or to hypophosphatasia.

PERFORMANCE CHARACTERISTICS

REP

PRECISION

Within Run: An abnormal specimen was run in replicate on one gel.

N = 15

	\bar{X}	SD	CV%
Liver	78.8 IU	1.0	1.2
Macrohepatic	21.2 IU	1.0	4.5

Run to Run: An abnormal specimen was run in replicate on five (5) gels.

N = 75

	\bar{X}	SD	CV%
Liver	78.2 IU	1.1	1.4
Macrohepatic	21.8 IU	1.1	4.9

CORRELATION

Studies were done on patient samples and controls using the TITAN GEL Alkaline Phosphatase (HR) method and the REP ALP method with better resolution and band integrity shown on the REP system.

REP 3

PRECISION

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

N = 15

	\bar{X}	SD	CV%
Liver %	62.4	1.1	1.8%
Bone %	37.6	1.1	3.0%

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

N = 120

	\bar{X}	SD	CV%
Liver %	61.0	1.9	3.0%
Bone %	39.0	1.9	4.8%

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REP Alkaline Phosphatase System

REP Alkaline Phosphatase Kit Cat. No. 3200

REP ALP Gels (10)
 REP Separation Enhancer (1.5 mL)
 REP ALP Reagent (10 x 45 mg)
 REP ALP Diluent (35 mL)
 REP Blotter A (10)
 REP Sample Cups (150)

Other Supplies and Equipment

REP	1352
REP 3	3700
GEL Alkaline Phosphatase Isoenzyme Control	5104
REP Prepper	1359
SUREPrep	1574
REP Prep	3100

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