TITAN GEL
Alkaline Phosphatase (HR)

Cat. No. 3058

Helena Laboratories

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

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 (fast liver) 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 inactivation[2], inhibition with amino acids[3-5], urea denaturation[3-4], and electrophoresis on agarose[6], paper[7], starch gel[8, 14, 15], polyacrylamide gel[9, 16] and cellulose acetate[10-12].

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

PRINCIPLE

The Helena agarose 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 isoenzyme 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 TITAN GEL agarose allows the separation of 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]

By combining sample pretreatment and high resolution techniques, the TITAN GEL system separates 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 three intestinal fractions, but the clinical significance of these has not been determined.

The enzyme activity is developed using BCIP as the substrate and AMPD as the phosphate acceptor.

REAGENTS

1. **TITAN GEL Alkaline Phosphatase (HR) Diluent**
   Ingredients: 2-Amino-2-Methyl,1-Propanol
   5-Bromo-4-Chloro-3-Indolyl Phosphate-p-Toluidine salt
   Magnesium Sulfate
   Sodium azide
   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. **TITAN GEL Alkaline Phosphatase (HR) Reagent**
   Ingredients: The concentration of the reactive ingredients after reconstitution is as follows:

2-Amino-2-Methyl,1-Propanol	2.0 M/L
5-Bromo-4-Chloro-3-Indolyl Phosphate-p-Toluidine salt	1.7 mM/L
Magnesium Sulfate	0.85 mM/L
NBT (nitro blue tetrazolium)	1.83 mM/L
Sodium azide	0.1%

   Preparation for Use: Reconstitute each vial with 3 mL of Alkaline Phosphatase Diluent
   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. **TITAN GEL Alkaline Phosphatase (HR) Gel**
   Ingredients: Each gel contains agarose in a barbital-sodium barbital buffer. 0.1% 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. 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.
   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. **TITAN GEL Alkaline Phosphatase (HR) Buffer**
   Ingredients: When dissolved, this buffer contains Tris-barbital-sodium barbital.

WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST. The buffer contains barbital which, in sufficient quantity, can be toxic.

Preparation for Use: Dissolve one package in 1000 mL purified water. The buffer is ready for use when all material is completely dissolved.

Storage and Stability: The packaged buffer should be stored at room temperature and is stable until the expiration date indicated on the package. Diluted buffer is stable for two months at 15 to 30°C.

Signs of Deterioration: Discard packaged buffer if the material shows signs of dampness or discoloration. Discard diluted buffer if it becomes turbid.

5. **Alkaline Phosphatase Separation Enhancer**

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

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 two bands if the enhancer is functioning properly.

INSTRUMENTS

Visual inspection for the presence of isoenzyme bands, indicating disease conditions, is sufficient. The bands may be scanned using any high quality scanning densitometer with visible capabilities such as the Helena CliniScan™2 (Cat. No. 1260) or the EDC® (Cat. No. 1370) may be used.

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 sample 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 TITAN GEL Alkaline Phosphatase (HR) Kit (Cat. No. 3058)

- TITAN GEL Alkaline Phosphatase HR Gels (10)
- TITAN GEL Alkaline Phosphatase HR Buffer (2 pkg)
- TITAN GEL Blotter A (20)
- TITAN GEL Blotter B (20)
- TITAN GEL Blotter D (10)
- TITAN GEL Wicks (40)
- TITAN GEL Alkaline Phosphatase HR Templates (10)
- TITAN GEL Alkaline Phosphatase Separation Enhancer (0.6 mL)
- TITAN GEL Alkaline Phosphatase HR Reagent (4 mg)
- TITAN GEL Alkaline Phosphatase HR Diluent (30 mL)

Materials provided but not contained in the kit:

GEL Alkaline Phosphatase Control	5104
Titan Plus Power Supply	1504
TITAN GEL Cooling Device	3039
Dialamatic Microdispenser and Tubes	6210/6211
TITAN GEL Chamber	4063
I.O.D.	5116
TITAN GEL Isoenzyme Incubation Chamber	4062

Materials needed but not provided:

Specimen sample cups - test tubes

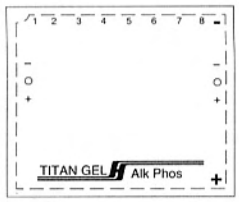
SUMMARY OF CONDITIONS

Gel	TITAN GEL Alk Phos Gel
Buffer Dilution	1000 mL
Buffer Volume	200 mL Total
Sample Volume	5.0 µL
Sample Absorption Time	10 minutes
Electrophoresis Time	30 minutes
Voltage	250 V
Incubation Time	30 minutes
Incubation Temperature	45°C
Drying Time	approximately 10 minutes at 50-60°C
Scanning Wavelength	595 nm

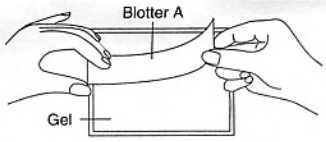
STEP-BY-STEP

A. Sample Preparation and Application

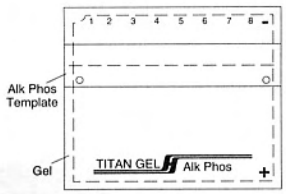
1. Prepare each sample and control by mixing 5 µL of Separation Enhancer with 25 µL of sample in small test tubes.



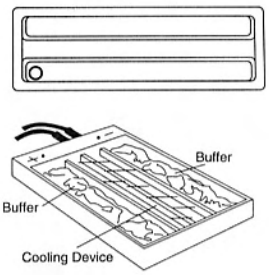
2. Immediately remove the gel from the protective packaging. One edge of the gel has been numbered for easy sample placement and identification.
3. Using a TITAN GEL Blotter A, quickly blot the gel at the area of application (the dashes (-) on the edges of the gel indicate the sample application alignment).



4. Place the template on the gel, aligning the application slits with the dashes (-), trying to avoid any air bubbles under it. Place a Blotter A over the template and remove any bubbles in the slit area with slight fingertip pressure. Retain the blotter for use in Step 9.

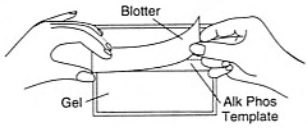


5. Place 5.0 µL of each sample on the template slits, spreading the sample completely over the entire slit. Apply the samples as quickly as possible.
6. Wait 10 minutes after the last sample has been applied. This allows the samples to diffuse into the agarose and the enhancer to react.
7. While the samples are absorbing, place a room temperature equilibrated cooling device in the center of the chamber. Wet the entire surface of the cooling device with a few drops of buffer.



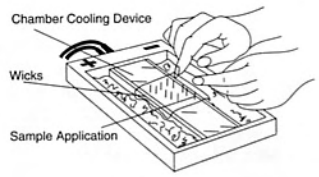
8. Pour 100 mL of buffer into each of the outer compartments of the electrophoresis chamber.

9. At the end of the 10 minute absorption time, gently blot the template with a Blotter A. Carefully remove the template.



B. Electrophoresis of the Gel

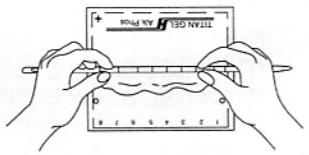
1. Quickly place the gel into the chamber, laying it agarose side up on top of the chamber cooling device. Position the gel so that the (-) sign on the gel is placed on the (-) side of the chamber. Avoid trapping air bubbles between the agarose and the glass of the cooling device. Run only one gel per chamber.
2. Prepare a wick for each side of the gel by placing two TITAN GEL Wicks together in two sets making two thick wicks. Evenly align the edges of each set of wicks while they are dry. Dip the wicks into the chamber buffer and remove excess buffer by squeezing them between two fingers. Then attach the wicks to each side of the gel parallel to the edge of the chamber cooling device. One edge of the wicks must be immersed in the buffer while the other edge makes contact with the edge of the agarose. Gently rub one finger across the wick at the gel contact area to insure good contact without bubbles.



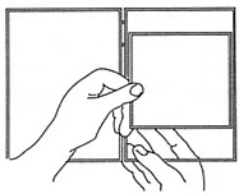
3. Place the cover on the chamber and allow the gel to remain in the chamber for 30-60 seconds before turning on the power. This will allow the gel to equilibrate in the buffer.
4. Electrophorese for 30 minutes at 250 volts.

C. Visualization of the Isoenzyme Bands

1. Reconstitute the Alkaline Phosphatase Reagent with 3 mL of Alkaline Phosphatase Diluent 10 minutes before the end of electrophoresis to allow for complete dissolution.
2. Remove the gel from the electrophoresis chamber and place, agarose side up, on a Blotter D. Blot the gel with a TITAN GEL Blotter B.



3. Place a 5 mL serological pipette lengthwise along the anode edge of the agarose gel. Dispense 1.0 mL of dissolved reagent on the gel in front of the pipette. Gently spread the reagent by slowly pulling the pipette across the agarose to the cathode edge being careful not to press down on the gel or to roll the reagent off the gel.
4. Count to 5 slowly. Add 1.0 mL reagent to the gel again. Then pull the pipette across the gel from the cathode to the anode edge in the same manner.
5. Count to 5 slowly, and spread the reagent across the gel a third time. Count to 5 again, and then pull the excess reagent off the gel on the cathode side.
6. Lift the gel off the blotter and place it into a preheated (45°C) TITAN GEL Isoenzyme Incubation Chamber containing a moist pad.



7. Place the Incubation Chamber in a Helena I.O.D. or other laboratory incubator at 45°C for 30 minutes.
8. After incubation, place the gel in a 10% acetic acid wash for 5 minutes with gentle agitation.
9. Remove the gel from the acetic acid wash and place on a Blotter D, agarose side up. Layer the following items on top of the gel in order: one Blotter B, three double medical tissues or towels, and an Isoenzyme Development Weight (Cat. No. 5014). Leave the gel for 5 minutes. At the end of the 5 minute period, carefully remove the weight, tissues and blotter. Rinse the gel in distilled water for 30 seconds.
10. Remove the gel from water and place it on a blotter pad agarose side up. Dry the gel at 50-60°C until the surface is clear and dry.

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.

Stability of End Product: Gels should be scanned and/or interpreted immediately. Store in the absence of light.

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

Quality Control: The GEL Alkaline Phosphatase (HR) 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 EDC® and CliniScan™2 Densitometers will automatically print the relative percent and the absolute values for each band. Refer to the Operator's Manual provided with the densitometer.

EXPECTED VALUES

Interpretation of isoenzyme patterns should not be attempted without knowledge of the total ALP level in the patient's serum. Each laboratory should establish its own normal range for the methodology it has selected. Serum from normal individuals may contain small amounts of liver, bone and intestinal ALP.[10, 12, 21] The macrohepatic band is normally in an undetectable level. ALP levels are age and sex dependent.[22-24] Fifty-two samples from supposedly healthy, adult men and women were used to obtain an expected range with the following results:

Liver	12.3	-	66.0 IU
Bone	15.0	-	62.4 IU
Intestine	0.0	-	2.2 IU

LIMITATIONS

The TITAN GEL Alkaline Phosphatase method is for semi-quantitative use only and should not be used to generate quantitative results. An assayed control should be run with each group of patients specimens. Patient scans and band intensities should be visually compared to the control data.

Linearity studies were done for total alkaline phosphatase and three of the more commonly seen bands. If specimens have higher concentrations than those given below, dilute and retest the specimens. Dilutions should be made so that the elevated band concentration falls within the appropriate linearity range.

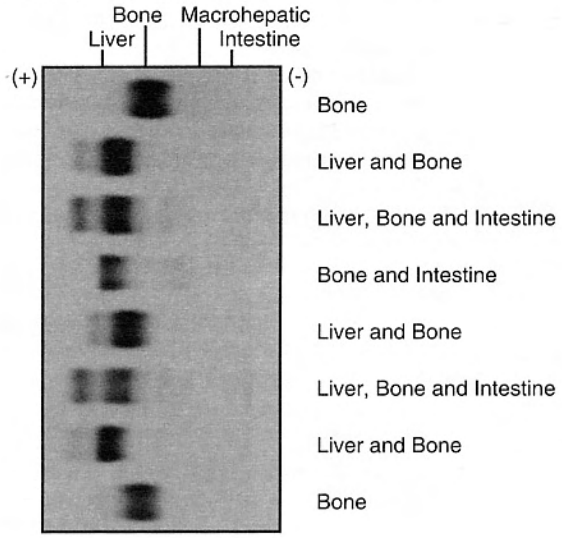
Component	Linear to
Total Alkaline Phosphatase	- 358 U/L
Liver band	- 171 U/L
Macrohepatic band	- 186 U/L
Bone band	- 123 U/L

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 macrohepat-

ic (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. Two of the intestinal bands migrate cathodic to the macrohepatic bands, and the third intestinal band migrates between the bone and macrohepatic bands. The intestinal bands are sharp and narrow. Albumin will appear as an anodic artifact. The major improvement between this method and existing methods is the vastly improved separation of bone and liver. This permits the identification of the bone isoenzyme within the normal alkaline phosphatase range.



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 associates[11] 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.

INTESTINAL ISOENZYME: Normally seen in the serum of subjects who have B or O blood types, especially after a fatty meal. Pathologically the band may be present in perforation of the bowel, ulcerative diseases of the intestine and faintly in liver cirrhosis as well as in intestinal perforation.[21, 22]

PERFORMANCE CHARACTERISTICS

Precision Studies

Within Run: A normal specimen was run in replicate on one gel. n = 8

	$\bar{X}$	SD	CV%
Liver	44.3 IU	0.7	1.6
Bone	55.7 IU	0.7	1.2

Run to Run: One normal specimen was run eight times on 8 gels.

	Bone	Liver
n	64	64
$\bar{X}$	55.7	44.3
SD	1.7	1.7
CV%	3.0	3.7

BIBLIOGRAPHY

1. Fishman, W.H., Am J Med, 56:617-650, 1974.
2. Posen, S. et al., Ann Int Med, 62(6):1234-1243, 1965.
3. Stepan, J. and Vecerek, B., Acta Univ Carol, Mono 77:135-140, 1977.
4. O'Carroll, D. et al., AJCP, 63:564-572, 1975.
5. Rhone, D.P. and Mizuno, F.M., AJCP, 59:531-541, 1973.
6. Sundblad, L. et al., Clin Chim Acta, 45:219-223, 1973.
7. Baker, R.W.R. and Pellegrino, C., Scan J Clin Lab Invest, 6:94-101, 1954.
8. Keiding, N.R., Scan J Clin Lab Invest 11:106-112, 1959.
9. Johnson, R.B., et al., Clin Chem, 18(2):110-115, 1972.
10. Fritsche, H.A. and Adams-Park, H.R., Clin Chem, 18(5):417-421, 1972.
11. Viot, M. et al., Biomedicine, 31:74-77, 1979.
12. Rhone, D.P. et al., Clin Chem, 19(10):1142-1147, 1973.
13. Moss, D.W. and Edwards, R.K., Clin Chim Acta, 143:177-182, 1984.
14. Robinson, J.C. and Pierce, J.E., Nature, 204:472-473, 1964.
15. Moss, D.W. et al., Biochem J, 98:32C-33C; 1966.
16. Rosalki, S.B. and Foo, A.Y., Clin Chem, 31(7):1198-1200, 1985.
17. Young, D.S. et al., Clin Chem, 21(5)246D-248D, 1975.
18. Ahmad, I., Clin Chem, 24(10):1850-1851, 1978.
19. Massion, C.G. and Frankenfeld, J.K., Clin Chem, 18(4):366-373, 1972.
20. Dysert, P., Personal Communication, May, 1981.
21. Lee, L.M. and Kenny, M.A., Clin Chem, 21(8):1128-1135, 1975.
22. Wolf, P.L., Arch Pathol Lab Med, 102:497-501, 1978.
23. Eastman, J.R., and Bixler, D., Clin Chem, 23(9):1769-1770, 1977.
24. Cherian, A.G. and Hill, J.G., AJCP, 70(5):783-789,1978.
25. Nerenburg, S.T., Medical Technology, Lea and Febiger Inc., Philadelphia 122-123, 1973.

TITAN GEL Alkaline Phosphatase (HR) System

TITAN GEL Alkaline Phosphatase (HR) Kit Cat. No. 3058
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- TITAN GEL Alkaline Phosphatase HR Reagent (4 mg)
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Other Supplies and Equipment

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I.O.D.	5116
TITAN GEL Isoenzyme Incubation Chamber	4062
Development Weight	5014

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