Alkaline Phosphatase Isoenzyme Procedure

Cat. No. 5102

The Helena Alkaline Phosphatase Isoenzyme Procedure is intended for the qualitative determination of serum alkaline phosphatase isoenzymes by electrophoresis on cellulose acetate.

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 physicochemical 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 placental isoenzymes, other ALP isoenzymes have been identified in serum. These include fast liver (pre-liver), Regan, Nagao, PA, and renal isoenzymes.

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], polyacrylamide gel[9] and cellulose acetate[10-12].

The Helena cellulose acetate method offers several distinct advantages over other identification methods. The technique is suitable for the electrophoretic fractionation of ALP in large numbers of sera, provides ease of handling, stability of supporting membranes and media, and reproducibility of results.

PRINCIPLE

The isoenzymes of alkaline phosphatase are separated according to their electrophoretic mobility on cellulose acetate in a tris-barbital-sodium barbital buffer. The colorimetric reaction occurs by hydrolyzing the indolyl dye.

REAGENTS

1. **Alkaline Phosphatase Indolyl Blue Reagent (Cat. No. 5102) Ingredients:** When reconstituted as directed, the concentration of the reactive ingredients is as follows:

5-Bromo-3-Indolyl Phosphate p-Toluidine Salt	8.35 mM
2-Amino-2-methyl-1-propanol	1 M
Magnesium Chloride	1 mM
Stabilizers	

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

   Preparation for Use: Reconstitute each vial of reagent with 3 mL of Diluent. Mix the reagent to obtain complete dissolution. The reagent may be used as soon as reconstituted or within 48 hours.

   Storage and Stability: The reagents should be stored at 2 to 8°C and are stable until the expiration date indicated on the label. The reconstituted reagent is stable 48 hours.

   Signs of Deterioration: The dry, unreconstituted reagent should be uniformly off-white to light lavender in color.

2. **Alkaline Phosphatase Indolyl Blue Diluent**

   Ingredients: 2-Amino-2-methyl-1-propanol and Magnesium Chloride

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

   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.

3. **Electra® HR Buffer (Cat. No. 5805)**

   Ingredients: The buffer is a tris-barbital-sodium barbital buffer. **WARNING: FOR IN-VITRO DIAGNOSTIC USE. DO NOT INGEST.** The buffer contains barbital which, in sufficient quantity, can be toxic.

   Preparation for Use: Dissolve one package in 750 mL deionized water. The buffer is ready for use when all material is completely dissolved.

   Storage and Stability: The packaged buffer should be stored at 15 to 30°C 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.

4. **Titan® III Plates (Cat. No. 3001)**

   Ingredients: Cellulose acetate

   WARNING: FOR IN-VITRO DIAGNOSTIC USE

   Preparation for Use: The plates are ready for use as packaged.

   Storage: The plates should be stored at 15 to 30°C.

SPECIMEN COLLECTION AND HANDLING

Specimen: Serum is the specimen of choice. Plasma collected in heparin may be used. Anticoagulants containing oxalate, citrate or EDTA cannot be used because these substances inhibit the alkaline phosphatase activity.[13]

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, 13, 18, 19]

Interfering Substances:

1. High concentrations of phosphate, oxalate, citrate and cyanide will inhibit ALP activity.[13, 18]
2. Excess glycine may inhibit ALP activity by complexing magnesium.[13]
3. EDTA inhibits some of the isoenzymes of ALP. Do not use as an anticoagulant.[13]
4. Several drugs cause an enzymatic imbalance which may change the ALP level.[13, 14]

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.[6, 15, 18]

PROCEDURE

Materials Provided: The following materials are necessary for use in the Alkaline Phosphatase Isoenzyme Test:

Item	Cat. No.
Super Z-12 Applicator	4090
Super Z-12 Sample Well Plate (2)	4096
Super CPK Aligning Base	4094
Titan Gel Chamber	4063
Dialamatic Microdispenser and Tubes	6210
1000 Staining Set	5122
Development Weight	5014

Bufferizer	5093
Titan® III Iso-Vis	3001
Electra® HR Buffer	5805
Alkaline Phosphatase Isoenzyme Control	5139
Alkaline Phosphatase Indolyl Blue Reagent	5102
Titan Blotter Pads	5037
Zip Prep	5090
Helena Marker	5000
Zip Zone® Chamber Wicks	5081
Glue Stick	5002
TITAN GEL Incubation Chamber	4062
Titan Plus Power Supply	1504

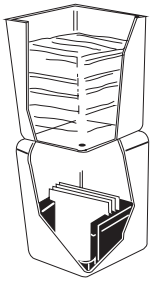
SUMMARY OF CONDITIONS

Plate	Titan® III Iso-Vis
Buffer	Electra® HR
Soaking Time for Plates	30 minutes
Sample Size	10 µL
Number of Applications	2-3
Electrophoresis Time	20 minutes
Voltage	180 V
Incubation Time	25 minutes
Incubation Temperature	37°C
Drying Time	10 minutes at approximately 56°C

STEP-BY-STEP METHOD

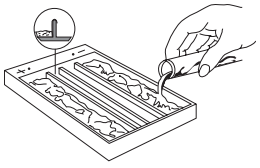
A. Preparation of Titan® III Plate

1. Properly code the required number of Titan® Plates by marking on the glossy, hard side with a Helena Marker. Place the mark in a corner of the plate.
2. Dissolve one bag of Electra® HR Buffer in 750 mL deionized water.
3. The plates should be soaked in the Bufferizer for 30 minutes according to the instructions for use. Alternately, the plates may be wetted by slowly and uniformly lowering a rack of plates into the buffer. The same soaking buffer may be used for soaking up to 12 plates, or for approximately one week if stored tightly closed. Improper storage may cause poor separation of the isoenzymes.



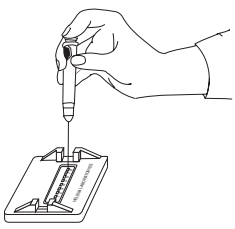
B. Preparation of Zip Zone® Chamber

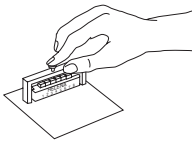
1. Pour approximately 100 mL of buffer into each of the outer sections of the chamber.
2. Wet two chamber wicks in the buffer and drape one over each support bridge, being sure it makes contact with the buffer and that there are no air bubbles under the wick.
3. Cover the chamber to prevent buffer evaporation. Discard the buffer after use.



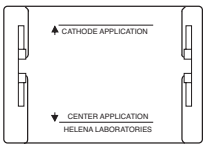
C. Sample Application

1. Place 10 µL of sample into each well of the Sample Well Plate using the Microdispenser. Cover the Sample Well Plate with a glass slide if the samples are not used within 2 minutes.
2. Prime the Super Z Applicator by quickly depressing the tips into the sample wells 3 or 4 times. Apply this loading to a piece of blotter paper. Do not load the applicator again at this point, but proceed quickly to the next step. Priming the applicator makes the second loading more uniform.





3. Remove the wetted Titan® III Plate from the buffer with the fingertips and blot once firmly. Place a drop of water on the center of the aligning base to prevent the plate from shifting during the superimposed applications. Place the plate in the Aligning Base, cellulose acetate side up, aligning the edge of the plate with the black scribe line marked "CENTER APPLICATION". The plate should be positioned so that the identification mark is always aligned with sample #1.



4. Apply the sample to the plate by depressing the applicator tips into the sample wells 3 or 4 times and promptly transferring the applicator to the Aligning Base. Press the button down and hold it 5 seconds. Make 2 to 3 superimposed applications by repeating this step.

D. Electrophoresis of Sample Plate

1. Quickly place the plate in the chamber cellulose acetate side down. Place a weight (glass slide, etc.) on the plate to insure contact with the wicks.
2. Electrophorese for 20 minutes at 180 volts.

E. Visualization of Isoenzyme Bands

1. Remove the sample plate from the chamber at the end of the electrophoresis period and blot lightly. Place the plate, cellulose acetate side up, on the blotter. Pipette 1.5 mL of the reagent onto the cellulose acetate surface. Tilt the blotter until the reagent covers the surface of the plate. Allow the reagent to soak into the plate for 1 minute.
2. Lay a clean glass rod or a serological pipette on the cellulose acetate, and gently roll it across the plate to remove any excess reagent. Failure to remove sufficient excess reagent or excessive pressure on the rod will cause smearing of the pattern.
3. Place the plate, acetate side up, into a preheated Incubation Chamber for 30 minutes at 37°C.
4. After incubation, place the plate in a staining rack, and immerse it in 5% acetic acid for 5 minutes.
5. Then, immerse the plate and rack in water for 5 minutes.
6. Remove the plate from the rack and lay it on a blotter. Dry it in a 56°C oven for 10 minutes.

F. Evaluation of the ALP bands

Qualitative evaluation: The ALP plates may be inspected visually for the presence of the isoenzyme bands.

Stability of end product: The plates should be viewed within the working day. Protect from light in the interim.

Quality Control: The Alkaline Phosphatase Isoenzyme Control (Cat. No. 5139) verifies all phases of the procedure and should be used on each plate run. The control may be used as a marker for the proper location of the bands.

RESULTS

Results of the alkaline phosphatase isoenzyme migration are described in comparison to typical serum protein migrations. Fast liver (pre-liver) migrates in the protein alpha$_1$ region and the major liver band migrates in the alpha$_2$ region. Placental, Regan, Nagao, and renal isoenzymes migrate with bone in the alpha$_2$/beta region, but they appear as tighter bands than bone. PA migrates cathodic to the intestinal band in the gamma region. An ultra-fast band which migrates in the albumin region is seen occasionally using the visualization method. It is believed to be caused by bilirubin bound to albumin.[29, 30] A specimen containing both liver and bone may exhibit one wide diffuse band in the alpha$_2$ pre-beta region. When such a pattern is obtained, heat inactivation may be helpful in differentiating the two isoenzymes.

Bone, being extremely heat sensitive, will be 90-100% inactivated while approximately 30% of the liver is affected. See the section entitled Further Testing and Special Treatment of the Serum Specimen for instructions for heat inactivation. Heat inactivation may also be used in differentiating bone from placental, Regan, or Nagao isoenzymes. These three isoenzymes are extremely heat stable and will not be inactivated by heat at 56°C for 10 minutes.

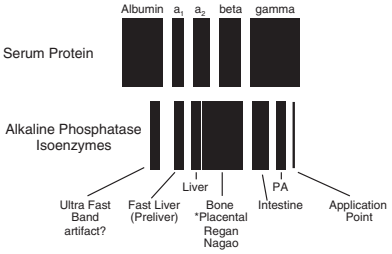


Figure 1: Schematic representation of the electrophoretic mobility of alkaline phosphatase isoenzyme compared to serum proteins.

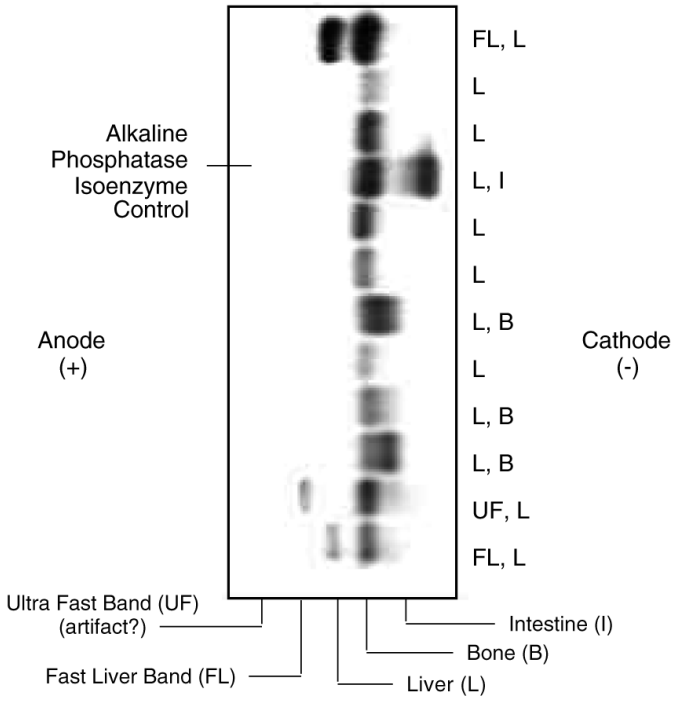


Figure 2: Helena Iso-Vis plate showing the migration patterns of the alkaline phosphatase isoenzymes.

Further Testing and Special Treatment of the Serum Specimen:

1. Total alkaline phosphatase activity should be determined on all samples. Interpretation of the isoenzyme pattern should not be made without knowledge of the total activity.
2. Heat inactivation[1, 2, 5] and chemical inhibition:[1, 3-5] Heat inactivation and chemical inhibition procedures used in conjunction with electrophoresis may be helpful in positively identifying the isoenzymes of ALP. After treatment with heat or chemicals, electrophoresis should be performed with the treated and untreated serum samples run side by side on the cellulose acetate electrophoresis plate. Should heat inactivation be desired, the following guidelines are offered:
   a. Place 0.5 mL of serum in a 12 x 75 mm test tube.
   b. Place the serum sample in a hot water bath for exactly 10 minutes. Some investigators have reported using 55 ± 0.1°C[16] while others have used 56 ± 0.1°C. The decision on the temperature of choice should be made in the individual laboratory and strictly adhered to at all times. Timing is also very critical. A timing device with a second hand should be used.
   c. After heating, immediately place the serum sample into ice.
3. Sample dilution:
   Specimens containing both bone and liver isoenzymes may exhibit one broad diffuse band in the alpha$_2$ pre-beta region because the bone and liver do not separate. Dysert[16] has reported good results in the separation of bone and liver isoenzymes by diluting serum specimens with saline (0.85%) so that the total ALP is no greater than 200 U. Both a diluted and non-diluted sample should be run side by side on the same cellulose acetate plate.

INTERPRETATION OF RESULTS

LIVER ISOENZYMES: The major liver isoenzyme (in the α_2 position) is the isoenzyme most frequently elevated when total ALP levels are elevated.[10, 12] The α_2 liver ALP increases in the blood early in liver disease before most other liver function tests show abnormalities. An extensive group of conditions lead to increased α_2 liver ALP including 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 to the liver.

Fast liver (in the α_1 position) 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 α_1 liver 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 fast liver 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.[21] An abnormally high bone isoenzyme level may also be indicative of bone cancer, osteomalacia or coeliac sprue. A decreased bone ALP in children may be attributed to cretinism or to hypophosphatasia.

PLACENTAL ISOENZYME: Appears in the serum of pregnant women late in the first trimester of pregnancy and may remain elevated for one month after termination of pregnancy.[22, 24] Infarction of the placenta in toxemia increases the serum placental isoenzyme.

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.[17, 18]

RENAL ISOENZYME: A rare isoenzyme reported by Nerenberg[21] and Kranc[25] which, like the Regan isoenzyme, migrates to the placental position. This isoenzyme represents a disease state of the kidneys or rejection of kidney transplant.

REGAN ISOENZYME: Isolated from the sera of patients with neoplasms.[1, 26, 27] Because of the similarities to placental isoenzyme, it has been referred to as carcinoplacental isoenzyme. Regan has been isolated from patients with lung cancer, breast cancer, ovarian cancer, and carcinoma of the colon.

NAGAO ISOENZYME: A variant of Regan isoenzyme that migrates in the same position as Regan on cellulose acetate.[1, 8] It has been isolated in metastatic carcinoma to the pleural surfaces and in adenocarcinoma of the pancreas or bile duct.

PA ISOENZYME: An unusual band observed in sera of patients with pancreatic cancer.[28] Cha observed the band in 16 patients (15 with cancer of the pancreas and one with hemochromatosis).

ULTRA-FAST BAND: A band migrating in the albumin position on cellulose acetate.[29, 30] Controversy exists as to the identification of this band. It may be an artifact caused by an albumin-bilirubin complex or by some other substance, or in some instances, it may be a

true isoenzyme. Further research must be conducted to determine its true origin and significance.

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