

SPIFE® Touch Alkaline Phosphatase (ALP) Isoenzyme Procedure

Cat. No. 3345, 3346

The SPIFE Touch Alkaline Phosphatase (ALP) Isoenzyme 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 SPIFE Touch 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 identified¹. 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 SPIFE Touch Alkaline Phosphatase method offers several advantages over all existing methods in that macrohepatic, liver, bone and intestine are all clearly separated.

PRINCIPLE

The SPIFE Touch Alkaline Phosphatase 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 mobility¹³. 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 isoenzyme¹³. 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 three 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. SPIFE Alkaline Phosphatase Gel

Ingredients: Each gel contains agarose in a tris-barbital-sodium barbital buffer with calcium lactate and preservatives.

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

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.

2. Alkaline Phosphatase 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 Alkaline Phosphatase Diluent.

Storage and Stability: The dry powder should be stored at 2 to 8°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. Alkaline Phosphatase 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 - IRRITANT. 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 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 on the bottle.

Signs of Deterioration: The diluent should not be used if it becomes milky white or shows signs of contamination.

4. SPIFE ALP Separation Enhancer (Cat. No. 3348)

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 8°C and is stable until the expiration date on the vial.

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

5. Citric Acid Destain

Ingredients: After dissolution, the destain contains 0.3% (w/v) citric acid.

WARNING: FOR IN-VITRO DIAGNOSTIC USE. 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 to 30°C. It is stable until the expiration date on the package.

Signs of Deterioration: Discard if solution becomes cloudy.

INSTRUMENTS

A SPIFE Touch Analyzer must be used to electrophorese and incubate the gel. The gel can be scanned on a densitometer such as the Quick Scan Touch/2000 (Cat. No. 1690/1660). Refer to the appropriate Operator's Manual for detailed operating instructions.

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 activity¹⁷. 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^{16,17,22,23}.

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 Mg²⁺¹⁷.
3. EDTA inhibits some of the isoenzymes of ALP. Do not use as an anticoagulant¹⁷.

- 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 SPIFE Alkaline Phosphatase Kit:

Sample Test Size	Cat. No.
40 sample	3346
20 sample	3345

SPIFE Alkaline Phosphatase Gels (10)
 Alkaline Phosphatase Reagent (10 x 4.5 mg)
 Alkaline Phosphatase Diluent (35 mL)
 SPIFE Blotter C (10)
 Citric Acid Destain (1 pkg)
 Blade Applicator Kit - 20 sample

Materials required but not contained in the kit:

SPIFE Touch Analyzer	1068
QuickScan Touch	1690
QuickScan 2000	1660
SPIFE ALP Separation Enhancer (1 x 4 mL)	3348
GEL Alkaline Phosphatase Isoenzyme Control	5104
REP Prep	3100
Gel Block Remover	1115
SPIFE Reagent Spreaders	3706
SPIFE Dispo Cups (Deep Well)	3360
SPIFE 20-100 Dispo Cup Tray	3366
Chamber Cover	8JP34012

STEP-BY-STEP

NOTE: If the staining chamber was last used to stain a gel, the SPIFE Touch software has an automatic wash cycle prompted by the initiation of the SPIFE Alkaline Phosphatase test. To verify the status of the stainer chamber, use the arrows under the **STAINER UNIT** to select the appropriate test, place the empty Gel Holder into the stainer chamber and press **START**. If washing of the staining chamber is necessary, the prompt "Vat must be washed. Remove gel and install gel holder." will appear. Press **RETRY** to begin the stainer wash. The cleaning process will complete automatically in about 7 minutes. To avoid delays after incubation, this wash cycle should be initiated at least 7 minutes prior to the end of the run.

I. Reagent Preparation

Reconstitute one vial of Alkaline Phosphatase Reagent with 3 mL of Alkaline Phosphatase Diluent and vortex well.

II. Sample Pretreatment

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

III. Sample Preparation

- If testing 21 to 40 samples, remove two Disposable Applicator Blades from the packaging. If testing fewer samples, remove only one Applicator Blade from the packaging.

- Place the two Applicator Blades into the vertical slots in the Applicator Assembly identified as 2 and 9. If using one Applicator Blade, place it into either slot 2 or 9.

NOTE: The Applicator Blade assembly will only fit into the slots one way; do not try to force the blade into the slots.

- Place an Applicator Blade Weight on top of each Applicator Blade. When placing the weight on the blades, position the weight with the thick side to the right.

- Slide two Disposable Cup strips into rows 1 and 3 of the Cup Tray.

NOTE: The sample will not migrate properly if samples are placed in rows 2, 4, or 5.

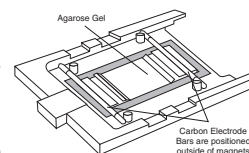
- Pipette 55 µL of pretreated patient serum or control into each cup. If testing less than 21 samples, pipette samples into the row of wells that corresponds with the Applicator Blade placement. Cover the tray until ready to use.

IV. Gel Preparation

- Remove the gel from the protective packaging and discard overlay.
- Place a REP Blotter C on the gel with the longer edge parallel with gel

blocks. Gently blot the entire surface of the gel using slight fingertip pressure on the blotter and remove the blotter.

- Dispense approximately 2 mL of REP Prep onto the left side of the electrophoresis chamber.
- 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 plastic gel backing, especially next to electrode posts, to remove excess REP Prep. Make sure no bubbles remain under the gel.
- Thoroughly wash the electrodes with deionized water before and after each use. Wipe the carbon electrodes with a lint-free tissue.
- Place the carbon electrodes on the outside ledge of the gel blocks, outside the magnetic posts. Improper contact between the electrode and the gel block may cause skewed patterns. Close the chamber lid.
- Use the arrows under **SEPARATOR UNIT** to select the appropriate test. To check parameters, select test and press **SETUP**.



V. Sample Application/Electrophoresis

Using the instructions provided in the Operator's Manual, set up the parameters as follows for the SPIFE Touch:

Due to variation in environmental conditions, *an Electrophoresis time of 27:00 minutes is recommended, but a range of 26:00 to 28:00 minutes is acceptable.

****a Dry time of 15 minutes is recommended, but a range of 10 to 20 minutes is acceptable.**

	Separator Unit
Load Sample 1	Prompt: None Time: 0:02 Temperature: 20°C Speed: 6
Load Sample 2	Prompt: None Time: 0:02 Temperature: 20°C Speed: 6
Load Sample 3	Prompt: None Time: 0:02 Temperature: 20°C Speed: 6
Load Sample 4	Prompt: None Time: 0:30 Temperature: 20°C Speed: 6
Apply Sample 1	Prompt: None Time: 1:00 Temperature: 20°C Speed: 6 Location: 1
Load Sample 5	Prompt: None Time: 0:30 Temperature: 20°C Speed: 6
Apply Sample 2	Prompt: None Time: 1:00 Temperature: 20°C Speed: 6 Location: 1
Load Sample 6	Prompt: None Time: 0:30 Temperature: 20°C Speed: 6
Apply Sample 3	Prompt: None Time: 1:00 Temperature: 20°C Speed: 6 Location: 1
Electrophoresis	Prompt: To Continue Time: 27:00* Temperature: 12°C Voltage: 430 V mA: 75 mA

Apply Reagent	Prompt: Remove Gel Blocks Temperature: 45°C Cycles: 10
Incubate	Prompt: None Time: 28:00 Temperature: 45°C
End	
	Stainer Unit
Destain 1	Prompt: None Time: 5:00 Recirculation: On Valve: 2 Fill, Drain
Destain 2	Prompt: None Time: 5:00 Recirculation: On Valve: 2 Fill, Drain
Wash	Prompt: None Time: 0:30 Recirculation: On Valve: 7 Fill, Drain
Dry	Prompt: None Time: 15:00** Temperature: 70°C
End	

1. Open the chamber lid and place the Sample Tray with samples on the SPIFE Touch. Align the holes in the tray with the pins on the instrument.
2. Place a reconstituted vial of reagent in the center hole of the reagent bar, ensuring that the vial is pushed down as far as it can go. Close the chamber lid.
3. Use the arrows under **SEPARATOR UNIT** to select the appropriate test. Press **START** and choose an operation to proceed. The SPIFE Touch will apply the samples, and beep when completed.
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 **CONTINUE** button to start electrophoresis. When completed, the unit will beep.
7. Open the chamber lid and remove the chamber cover.
8. With the gel still in the chamber, use a Gel Block Remover to completely remove and discard the two gel blocks.
9. Use a lint-free tissue to wipe around the edges of the gel, including the gel block area.
10. Place two Reagent Spreaders (glass rods) on the gel, inside the magnetic posts. Close the lid.

V. Incubation

1. Press the **CONTINUE** button to apply reagent and then begin incubation.
2. At the end of the incubation, the instrument will beep. Remove the gel from the chamber.
3. 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.
4. Place the Gel Holder with the attached gel facing backwards into the stainer chamber.
5. Use the arrows under **STAINER UNIT** to select the appropriate test. Press **START** and choose an operation to proceed. The instrument will destain and dry the gel.
6. When the gel has completed the process, the instrument will beep. Remove the Gel Holder from the stainer and scan the bands.

Evaluation of the Alkaline Phosphatase Isoenzyme Bands

Gels should be visually evaluated for band positions using a bone/liver control. Scan the dried Alkaline Phosphatase gels by placing the gel in the QuickScan Touch/2000 using slit 4 and acid violet settings. The "Smoothing" function should be set on "1" to scan the patterns. 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. Protect gels from 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 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. Additional control may be needed for federal, state or local regulations.

Calculation of the Unknown

The Quick Scan Touch/2000 will automatically calculate and print the relative percent and the absolute values for each band. Refer to the appropriate 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 dependent²²⁻²⁵.

In children and adolescents, the bone isoenzyme is approximately 85% of the total ALP isoenzyme level. At 18-30 years of age, the bone isoenzyme decreases to 60% of the total; and above 30 years of age, it decreases to 40%²⁵. As the patients' age increases, the bone isoenzyme level decreases and the liver isoenzyme level increases.

Forty-two samples were obtained from supposedly normal, non-fasting adult men and women and were used to derive an reference range with the following results:

Liver	26.0 - 86.2%
Bone	10.7 - 68.3%
Intestine	0.0 - 15.9%

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.

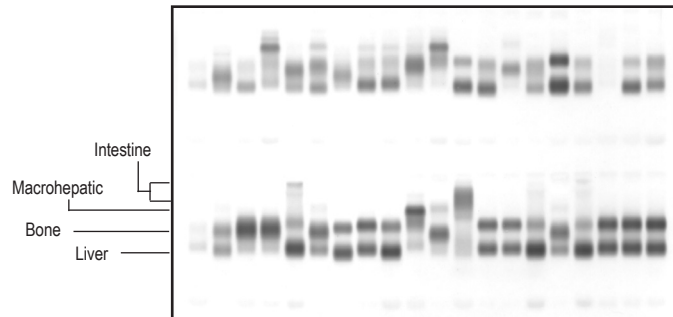


Fig 1: A SPIFE Alkaline Phosphatase gel showing relative position of the bands.

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 later stages of pregnancy, the placental band may migrate with the bone band. The placental band is heat stable and can be separated from bone.²⁷ 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 three 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 liver²².

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 state¹¹.

BONE ISOENZYME: This isoenzyme is elevated as a result of increased osteoblastic activity. It 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 rickets²⁶. An abnormally high bone isoenzyme level may also be indicative of bone cancer, osteomalacia or coeliac sprue²². A decreased bone ALP in children may be attributed to cretinism or to hypophosphatasia.

INTESTINE ISOENZYME: The intestinal band is detectable in about 20% of serum samples tested. The level is usually < 20% of the total alkaline phosphatase.²⁸ The intestinal band is most frequently noted in patients with blood groups O and B who are secretors of the H-blood group substance. It is elevated in these patients postprandially and after a fatty meal²⁸. Additionally, the intestinal band is found in disease states such as cirrhosis of the liver, malignancy, diabetes and chronic renal failure^{28,29}.

PLACENTAL ISOENZYME:²⁷ During pregnancy, most of the major serum enzyme concentrations remain unchanged. However, alkaline phosphatase increases during the later stages of pregnancy. A progressive rise begins about 32 to 34 weeks gestation, with relatively inconsistent values prior to that. The isoenzyme is heat stable and readily identifiable in the lab. If the heat stable isoenzyme value is low, the prognosis for the fetus is ominous. Little information is gained if the value falls within the normal range.

PERFORMANCE CHARACTERISTICS

PRECISION

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

N = 14

	\bar{X}	SD	CV%
%Liver	54.3	1.3	2.3
%Bone	45.7	1.3	2.8

Run to Run: A specimen was run in replicate on nine (9) gels.

N = 126

	\bar{X}	SD	CV%
%Liver	54.4	2.7	4.9
%Bone	45.6	2.7	5.9

CORRELATION:

Studies were done using 34 normal and abnormal patient samples and controls comparing the SPIFE Alkaline Phosphatase method and the SPIFE Touch Alkaline Phosphatase method on the SPIFE Touch.

N = 36

$$Y = 0.9632X - 1.2671$$

$$R = 0.9958$$

$$X = \text{SPIFE Alkaline Phosphatase on SPIFE 3000}$$

$$Y = \text{SPIFE Alkaline Phosphatase on SPIFE Touch}$$

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SPIFE Alkaline Phosphatase System

Cat. No.
3345, 3346

SPIFE Alkaline Phosphatase Kit

- SPIFE Alkaline Phosphatase Gels (10)
- Alkaline Phosphatase Reagent (10 x 4.5 mg)
- Alkaline Phosphatase Diluent (35 mL)
- SPIFE Blotter C (10)
- Citric Acid Destain (1 pkg)
- Blade Applicator Kit - 20 sample

Other Supplies and Equipment

SPIFE Touch	1068
QuickScan Touch	1690
QuickScan 2000	1660
SPIFE ALP Separation Enhancer (1 x 4 mL)	3348
GEL Alkaline Phosphatase Isoenzyme Control	5104
Gel Block Remover	1115
SPIFE Reagent Spreader	3386
REP Prep	3100
SPIFE 20-100 Dispo Cup Tray	3366
SPIFE Dispo Cups (Deep Well)	3360
SPIFE Reagent Spreaders	3706
Chamber Cover	8JP34012

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