1530 Lindbergh Dr • Beaumont, TX 77707-4131
Local 409-842-3714 • Toll free 800-231-5663 • www.helena.com
Email (Orders) orderentry@helena.com (General) helena@helena.com

Technical Bulletin - Alkaline Phosphatase Isoenzymes

When sample is pretreated with neuraminidase and run on the Helena gel formulation, it will separate out four alkaline phosphatase isoenzymes; liver, bone, macrohepatic (sometimes referred to as fast liver, or L2), and three intestinal bands. However, it must be emphasized that identification of alkaline phosphatase isoenzyme through electrophoresis alone is not definitive. There are multiple potential isoenzymes migrating in the bone/liver area that may cause interferences or affect quantitation when using electrophoretic methods alone (i.e. placental, Regan, Nagao, Pancreatic (PA) and renal isoenzymes). Additional testing or sample treatment is required for identification.

Historically, many labs have used isoenzyme electrophoresis as one component of an algorithm, which included other chemical or kinetic methods, and a variety of sample treatment protocols. Although specific recommendations regarding sample treatment protocols are not defined in our current procedures, a summary of isoenzyme characteristics and behaviors is provided below, and references to sample treatment protocols are available upon request.

Alkaline Phosphatase (ALP) Isoenzymes

Liver

The liver band migrates the most anodally (fastest) 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 (also referred to as fast liver, L2) band. Without enhancer, bone and liver will both migrate faster and will migrate closer together.

Bone

In the presence of a high concentration of bone activity, the bone will migrate slower than that of a normal patient. Bone and liver will appear more separated in this situation.

Intestinal

There are up to three intestinal bands. These are the slowest migrating bands and will be found closest to application. GGT elevation is a strong negative predictor of intestinal elevation. The easiest way to confirm an intestinal band would be to run with and without enhancer. If enhancer has no effect on migration, the band is likely intestinal and not macrohepatic.

Macrohepatic

Macrohepatic should not be confused with an immune complex, it is named “macrohepatic’ due to the size. It is also referred to as Liver 2, and fast liver. The term ‘fast liver’ refers to the migration of macrohepatic on cellulose acetate plate (the migration patterns of ALP isoenzymes differs between agarose and cellulose acetate). If there is a macrohepatic band present, there is usually a liver band. GGT is likely to be elevated.

Immune-complex

Immune complexes may also be called Macro-alkaline phosphatase, not to be confused with ‘macrohepatic/Liver 2/Fast liver’. Immune complexes stay at application and are not affected by enhancer. Immune complexes are partially heat stabile and smeary in appearance. Ficin or trypsin sample treatment may be useful.

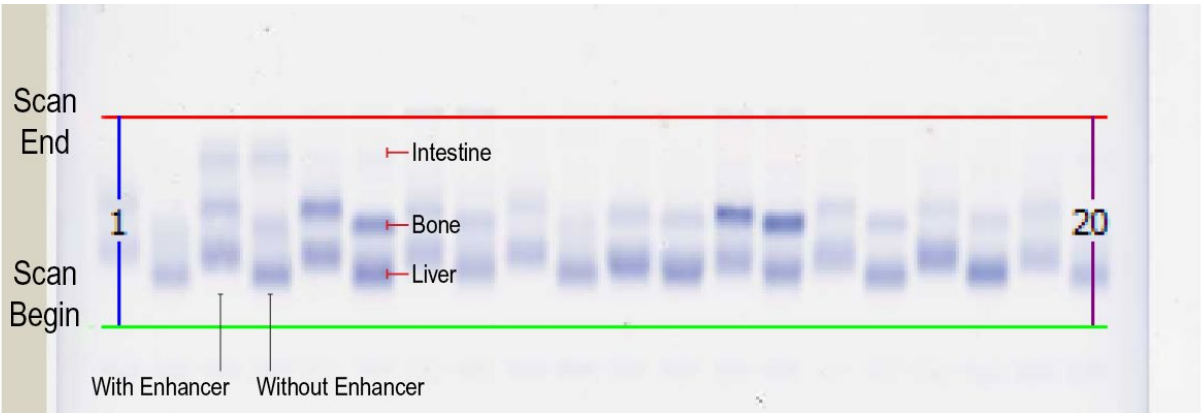
Placental

There are usually two placental ALP bands but can be up to four; one in bone/on the cathodal edge of bone, and one cathodal to bone. There could be up to three in bone.[1] Placental is affected by enhancer and moves cathodally with enhancer. Placental ALP (as well as neoplacental) is the most heat stable and can be confirmed with heat treatment. Placental ALP can also be differentiated with anti-placental antibody or via chemical means (see below).

Be aware that placental or neoplacental isoenzymes may be masked by bone on the SPIFE platform. Placental ALP can be elevated in some germ cell tumors, including seminomas and ovarian cell cancers. There are other, less common isoenzymes that may also migrate in similar regions and be masked by liver or bone. In addition, there may be variability between individuals and in disease states in migration characteristics, heat stability, and chemical inhibition.

Scanning Tips

1) Gels should be scanned immediately after being run.
2) Samples should be scanned from liver to bone to intestine.
3) Use a "Smoothing" function setting of 1, slit 4, and acid violet settings.
4) Baselining is acceptable – in moderation.
5) The delete function may be used to remove extra material in front of liver.
6) Rescan as needed, moving the scan begin and end.
7) If no valley between peaks is identified, use the reference overlay and best judgement for editing.

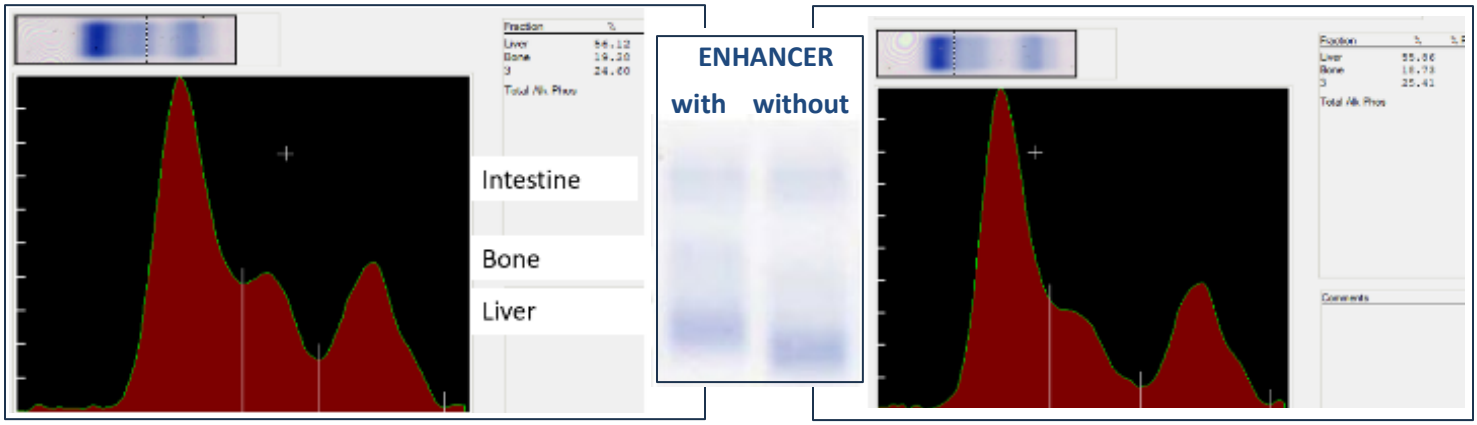


Sample Treatments

Dilution, comparing samples run with and without separation enhancer, heat inactivation, and chemical inhibition procedures used in conjunction with electrophoresis may be helpful in positively identifying the isoenzymes of ALP. After sample treatment, electrophoresis should be performed with the treated and untreated serum samples run side by side.

1. **Neuraminidase (Separation Enhancer)**
    a. Helena Laboratories does not require that all samples be run in duplicate (with and without neuraminidase). Running a sample with and without enhancer side by side is a useful practice in that the effect of enhancer varies between isoenzymes. The greater the sialylation, the more effect the enhancer will have.
    b. Bone is the most slowed by enhancer, macrohepatic the least. The relative effect of enhancer is bone > placenta > liver > macrohepatic.

c. Without enhancer, bone and liver will migrate closely together. (See image to the right) As the effect of enhancer is greater on bone than on liver, bone and liver will be more clearly separated when enhancer is used as seen on the scan to the left).
d. Enhancer has no effect on the migration of intestinal ALP.



Same patient sample run with Neuraminidase Separation Enhance (left) and without (right).

2. Dilutions

a. Helena SPIFE ALP Linearity: Samples are linear up to 250 U/L per isoform band. As the relative percentages of each band are unknown prior to electrophoresis, initial dilution protocol decisions must be based on total alkaline phosphatase activity. Samples with a total >450 U/L should always be diluted. To avoid the need to repeat samples, labs may choose to routinely dilute samples with a lower total activity. (200-250 U/L)
b. Dilution may affect mobility. This effect is due to different total protein concentration in the diluted sample. Dilution with saline has the greatest effect on mobility. Dilution has also been performed with heat inactivated serum, 7% albumin or DI water. Albumin or heat inactivated serum may have less effect on migration than saline.[2]
c. It may be helpful to run a diluted control next to diluted sample for visible comparison.
d. Be sure to dilute samples prior to the addition of enhancer.

3. Heat differentiation[3,4]

a. Placental and neoplacental (Regan, Nagao) are the most heat stable. Bone is the least heat stable. The relative sensitivity to heat is as follows: bone > liver > intestine > placental/neoplacental.
b. After 56°C for 10 minutes, 90% of placental and neoplacental activity will remain, approximately 50% of liver and intestinal, and 10% of bone with some variability.
c. After 5 minutes at 65°C, only placental or neoplacental will remain.
d. There are multiple protocols published for heat treatment with slight variations in temperature or timing. It is important to be consistent, use a timer, water bath, and plunge the heated sample into an ice bath immediately after incubating.
e. Run heat treated sample next to non-heat treated sample.
f. Complete heat treatment prior to the addition of neuraminidase.
g. If using heat treatment at 56°C, heat an additional specimen containing both bone and liver as a control to be run alongside the heated and non-heat treated test specimen.

h. Example heat treatment protocol
    i. Place 0.5 mL of serum in a 12 x 75 mm test tube.
    ii. Place the sample in a hot water bath.
    iii. Use 65°C for 5 minutes to evaluate for placental, 56°C for 10 minutes to differentiate between liver and bone.
    iv. As soon as the heat step has finished plunge the tube into an ice bath to stop inactivation.
    v. Electrophoresis should be performed side by side on the heated and unheated samples after neuraminidase treatment. As a control, process a specimen containing both liver and bone fractions so you can compare the effectiveness of the heat inactivation.

4. Cellulose Acetate

a. Catalog # 5139 Alkaline Phosphatase Isoenzyme Control contains bovine liver/intestine isoenzymes and is intended for use with the cellulose acetate platform. The intestinal band will migrate with human intestine with and without enhancer. **The bovine liver band will not migrate with human liver after treatment with enhancer.**
b. Neuraminidase is not used with the cellulose acetate platform.
c. The migration of bands is slightly different on the cellulose acetate platform, most notable is that macrohepatic (fast liver) migrates on the anodal side of liver.
d. The Helena Gel Alk Phos Isoenzyme Control for agarose gel (Catalog # 5104) contains only human liver and bone isoenzymes.

5. Amino Acid inhibition

The L isomers of various amino acids have been useful in differentiating isoenzymes due to their preferential inhibition effects.

a. 0.005M-L-phenylalanine inhibits intestinal and placental ALP to a greater extent than liver and bone.[5,6]
b. L-homoarginine: Liver and Bone are inhibited to a greater extent than intestine and placenta.[7]
c. L-tryptophan inhibits placental and intestinal alkaline phosphatase to a greater degree than liver and bone.[7]
d. L-Leucine can be used to differentiate Regan and Nagao; (Placental ALP and Nagao are sensitive to L-Leucine).

6. Ficin

Ficin can be useful for immune complexes. Add 5 mg of ficin to 100 microliters of serum and incubate 30 minutes at 37°C. Apply original sample and treated sample and run side by side.

7. Trypsin

Incubate with Trypsin at 37°C for 2 hours to resolve immune complex at application. Apply original sample and treated sample and run side by side.[8]

8. Urea

a. Urea at low concentrations (< 3mol/L) results in a reversible instantaneous inhibition. At higher concentrations inhibition is irreversible. Bone is the most affected, and placenta is the most resistant. (Order of inhibition: Bone > kidney > liver> intestinal > placental)
b. Fennelly et al found that when incubating with 4M urea there was less than 10% of liver and bone activity remaining, but 50% placental.[9]
c. Johnson et al used 1 volume of 6 mol/L urea to 1 volume serum, incubating for 18 min at 37°C to inhibit bone.[10]

9. **Anti-placental antibody**
    a. Polyclonal rabbit anti-placental antisera: Mix 100 microliters of serum with 20 microliters of antisera and apply immediately.
    b. Renal and intestinal are also affected by anti-placental antibody.

10. **Lectin**
    Wheat germ lectin (WGA) can be used similarly to neuraminidase to separate bone from liver, but functions in a different manner. The lectin reacts with bone, precipitating out at or near application. This allows a placental band to be more easily identified; however this has the disadvantage of lack of specificity,[11] and is considered inferior to heat inactivation for quantitation of bone.[12,13,14] With the use of lectin, bone quantitation is systemically underestimated relative to other methods[15,16,17] and is not recommended for patients with suspected liver disease or mixed profiles.[18]

References

1. Van Hoof VO, Lepoutre LG, et al. Improved agarose electrophoretic method for separation alkaline phosphatase isoenzymes in serum. Clin Chem. 1988;34(9):1857-1862
2. Ji, L et al Helena Electrophoresis kit for alkaline phosphatase isoenzymes. Abstract 075: Clin Chem 40 (6) p 1120 (1994)
3. Fishman, W.H., Perspectives on Alkaline Phosphatase Isoenzymes. Am J Med, 56:617-650, 1974.
4. Posen, S et al., Heat inactivation in the study of human alkaline phosphatase. Ann Int Med., 62, 1234-1243, 1965.
5. Wilkinson JH: Isoenzymes. 2nd Ed. Lippincott, Philadelphia, 1970
6. Shephard MDS et al; Quantitative method for determining serum alkaline phosphatase isoenzyme activity II. Development and clinical application of method for measuring four serum alkaline phosphatase isoenzymes. J Clin Pathol. 1986; 39:1031-1038
7. Lin CW et al. L-Homoarginine: An organ –specific uncompetitive inhibitor of human liver and bone alkaline phosphohydrolases. J Bio Chem. 247(10) May 1982 (3082-3087)
8. Buttery JE et al. Detection of alkaline phosphatase/ Immune complexes. Clin Chem 26 (11) p1620-1621 (1980)
9. Fennelly J et al. Value of differential thermostability, urea inhibition and gel filtration of alkaline phosphatase in identification of disease states. Gut, 1969, 10 45-51
10. Johnson R et al. A study of various electrophoretic and inhibition techniques for separating serum alkaline phosphatase isoenzymes. Clin Chem 19(2) 1972 110-115
11. Schreiber, WE and Whitta L, Precipitation of serum proteins by the lectin from wheat-germ, Clin Chem 1987 Jan 33 (1): 185-6
12. Farley JR. Quantification of skeletal alkaline phosphatase in osteoporotic serum by wheat germ agglutinin precipitation, heat inactivation, and a two-site immunoradiometric assay. Clin Chem 1994 40(9) 1749-1756 (1994)
13. Farley JR et al. Reference standards for quantitation of skeletal alkaline phosphatase activity in serum by heat Inactivation and lectin precipitation. Clin Chem 39(9) 1878-1884 ,1993.
14. Garrido JC et al. Comparison of three bone alkaline phosphatase quantitation method. Clin Chem 30 (p 1165-1166. 1992
15. Hodel SD et al, Comparison of alkaline phosphatase isoenzyme methods. Clin Chem July 2003 49. 6S Abstract D-96.
16. Garrido JC et al. Comparison of three bone alkaline phosphatase quantitation methods. Clin Chem 30 (p 1165-1166. 1992.
17. Black J and Oldfield S. Is it liver or is it bone? Abstract 0698: Clin Chem 40 (6) p 1120 (1994)
18. Day AP, Saward S, Royle CM, et al; Evaluation of two new methods for routine measurement of alkaline phosphatase isoenzymes. J Clinical Pathology 1992; 45:68-72

For more information, contact Technical Services by calling 409-842-3714 or 800-231-5663 (USA) or by email at technicalservice@helena.com.

399-048
9/25