Laboratory Procedure Manual

Analyte:	**Bone alkaline phosphatase**
Matrix:	**Serum**
Method:	**Hybritech Tandem-MP Ostase ImmunoEnzymetric**
Method No.:	
Revised:	
as performed by:	*Department of Laboratory Medicine* *University of Washington Medical Center*
Contact:	*Dr. Mark Wener, M.D.. Director*

Important Information for Users

The University of Washington periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for NHANES 2001–2002 data. There were two methods used to measure Bone Alkaline Phosphatase in NHANES 2001-2002. For NHANES 2001, the HybritechTandem-MP Ostase ImmunoEnzymetric assay was used for quantitative measurement of Bone Alkaline Phosphatase (BAP). For NHANES 2002, the Beckman Access Ostase assay was used to measure serum Bone Alkaline Phosphatase.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
l11_b	LBXBAP	Bone alkaline phosphatase (μg/L)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The Tandem-MP Ostase ImmunoEnzymetric Assay is an *In Vitro* device for the quantitative measurement of Skeletal Alkaline Phosphatase (sALP), an indicator of osteoblastic activity, in human serum. This device is intended to be used as an aid in the management of postmenopausal osteoporosis and Paget's disease.

The Ostase Assay is a solid phase, monoclonal antibody immnumoenzymetric assay. Samples containing sALP are reacted with a solution containing a biotin-labeled, sALP-specific monoclonal antibody. The reaction takes place in a plastic well strip (solid phase) coated with strepavidin and enclosed in a plastic frame. Following the formation of a solid phase/capture antibody/sALP complex, the microplate is washed to remove unbound sALP and is then incubated with an enzyme substrate. The amount of substrate turnover is determined colorimetrically by measuring the absorbance of the quenched reaction at 405 nm in a microplate reader. The absorbance is proportional to the concentration of sALP present in the test sample. The calculation of sALP concentration in the sample is based on concurrent testing of sALP Calibrators and the Zero Diluent/Calibrator.

Serum levels of sALP are believed to reflect the metabolic status of osteoblasts. An accurate assessment of bone metabolism is critical for determining the severity of metabolic bone disease and responses to therapy. Measurement of serum levels of sALP has been shown to be useful in evaluating patients with Paget's disease, osteomalacia, primary hyperparathyroidism, renal osteodystrophy, osteoporosis and metastases to bone. Total alkaline phosphatase determinations have been the accepted method for the diagnosis and monitoring of patients with Paget's disease.

Paget's disease of bone is a common skeletal disorder in which there is a focal proliferation of the normal cellular components of bone. Paget's disease is more prevalent than once thought with the prevalence rate in certain populations 3%-4% in middle-aged patients and 10%-15% in the elderly. This disease does not affect young individuals. The majority of patients with Paget's disease has no symptoms and often goes undiagnosed unless an abnormal X-ray or serum alkaline phosphatase level is found in the course of a medical evaluation for unrelated reasons. The most common complaints in symptomatic patients are pain and deformity.

The risk of osteoporosis, another bone remodeling disorder, depends in part upon skeletal development, the attainment of peak bone mass, and in later life, the amount of bone lost. In healthy children, bone formation is favored over bone resorption, which results in bone development and normal skeletal growth. In healthy young adults, bone formation and bone resorption are balanced, resulting in no net increase or decrease in skeletal mass. With advancing age, men and women experience and imbalance in bone remodeling in which resorption is slightly greater than formation, resulting in a continuous net loss of bone mass with time. If this imbalance persists, bone mass may decline until the skeleton is insufficient to withstand normal mechanical stresses, and it become abnormally susceptible to fractures. The excessive loss of bone mass with an increased susceptibility to fractures is a disorder known as osteoporosis.

The most common form of osteoporosis occurs in postmenopausal women and is the result of estrogen deficiency. Rapid bone loss accompanies the decline of estrogen

levels at the onset of menopause or as a result of surgical removal of the ovaries. Rapid bone loss occurs as a result of the combined effects of imbalance in bone remodeling and an increase in bone turnover. In the United States, osteoporosis affects some 25 million postmenopausal women and is the cause of approximately 1.5 million fractures annually, including approximately 500,000 vertebral crush fractures, 250,000 hip fractures, and 200,000 distal radius fractures.

Hormone replacement therapy is currently the most widely prescribed therapy for the prevention of osteoporotic fractures in postmenopausal women. However many women cannot, or will not, avail themselves of hormone replacement therapy because of the potential for the increased risk of cancer and the resumption of menstrual bleeding. For this reason, other compounds such as bisphosphonates, a standard treatment for Paget's disease of bone, have been developed to treat osteoporosis. The anti-resorptive properties of bisphosphonates decrease bone remodeling and, consequently, decrease the overall loss of bone.

Biochemical markers are useful in monitoring metabolic bone disease. Urinary hydroxyproline and total serum alkaline phosphatase have been used for monitoring the treatment of Paget's disease. Osteoporosis, however, represents a more subtle modification of the bone resorption process; therefore, more specific and sensitive markers are needed.

Osteomalacia is the term used to describe a pathological condition in bone in which the osteoid matrix (the proteinaceous scaffolding in bone) remains uncalcified. The most common condition causing osteomalacia is vitamin D deficiency, resulting in rickets in children or osteopenia with bone fractures in adults. Elevated serum alkaline phosphatase is a hallmark of this condition. Recent data suggests that mild degrees of vitamin D deficiency may be very common in the population, and could contribute to osteopenia in patients diagnosed with osteoporosis.

The Ostase assay is an In Vitro device for the quantitative measurement of skeletal alkaline phosphatase (sALP) in human serum. Changes in sALP have been shown to be useful in patients undergoing therapy for metabolic bone disorders. It may also be an indicator of vitamin D deficiency in some patients.

2. SAFETY PRECAUTIONS

Consider all samples received for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions. Wear gloves, lab coat, and safety glasses when handling all human blood products and infectious viruses. Place disposable plastic, glass, paper, and gloves that contact blood in a biohazard bag or discard pan to be autoclaved. Disinfect all work surfaces with a 1:200 dilution of Staphene (Calgon Vestal Laboratories, St. Louis, Missouri) Dispose diluted specimens and any other potentially contaminated materials in a biohazard bag at the end of the analysis to be autoclaved prior to final disposal. Autoclaved or disinfect other non- disposable material at the end of the working day.

Do not pipette by mouth. Do not eat, drink or smoke in designated work areas. Wash hands thoroughly after removal of personal protective devices used in handling specimens and kit reagents.

Material safety data sheets for all reagents used in the performance of this assay, including but not limited to Staphene, sodium hydroxide, sodium hypochlorite, and sodium azide, are kept in the Immunology Division, University of Washington Medical Center (UWMC).

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

A. Each shipment of specimens received from the NHANES IV mobile unit arrives with a corresponding transmittal sheet and a Send File (a comma delineated text file) transmitted electronically (labeled *boxnum.*shp). This file contains the following information:

Field	Type
Sample ID	XXXXXXXXX
Slot Number	XXX
Sample Collection Date	mm/dd/yyyy hh:mm:ss
MEC Comment Code	XX

B. The information from the shipping file is imported into a result file with the following format:

Field	Format	Type	Item ID
Sample ID	XXXXXXXXX	Int	
Slot Number	XXX	smallint	
Sample Collection Date	mm/dd/yyyy hh:mm:ss	Smalldatetime	
MEC Comment Code	XX	Smallint	
Bone Alk. Date of Receipt	mmddyyyy	Smalldatetime	LBXBAPDR
Bone Alk. Run num	{test code}mmddyy. x(letter)	Char(10)	LBXBAPBT
Bone Alk. Date of Analysis	mmddyyyy	Smalldatetime	LBXBAPDA
Bone Alk. Result	XXXXX.X	Numeric(6,1)	LBXBAP
Bone Alk. Comment	XX	Smallint	LBXBAPLC
Bone Alk. Analyst id	XXX	Char(3)	LBXBAPTK
Bone Alk. 2.5% repeat	XXXXX.X	Numeric(6,1)	LBCBAP

C. After the testing is completed, the run number, date of analysis, sALP result, sALP comment, sALP analyst, and the sALP 2.5% repeat results are entered into the result file.

D. Data entry is checked for errors.

E. After the sALP testing has also been completed, resulted, and checked, the result file is stored as a comma delineated file and is transmitted electronically to NHANES WESTAT. Electronic and hard copies of the files and all primary data are kept in the laboratory.

F. Technical support for this system is provided by Westat, Rockville, MD (1-301-294-2036)

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

A. No special preparation of the patient is necessary.

B. A whole blood specimen should be obtained by acceptable medical technique. Allow the blood to clot and separate the serum by centrifugation

C. The requested sample volume for the assay is 1.0 mL, and the minimum sample volume is 0.3 mL. 50 ∗L of serum per well is required

D. SERUM IS REQUIRED FOR THE TANDEM-MP OSTASE ASSAY. Plasma samples should **not** be used.

E. If the serum sample is to be assayed within 24-48 hours after collection, the specimen should be stored in a refrigerator at 2°C to 8°c.

F. Specimens held for longer times (up to 2 months) should be frozen at –70°C

G. Specimens should be collected in such a way as to avoid hemolysis.

H. Turbid serum samples or samples containing particulate matter should be centrifuged prior to use. Contamination or introduced particulate matter can lead to erroneous results.

I. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

J. Avoid repeat freeze/thaw cycles.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. PREPARATION OF REAGENTS, CALIBRTORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

A. Reagents and standard materials.

(1) ANTIBODY SET - Tandem-MP Ostase ImmunoEnzymetric Assay, cat no #4605 (Hybritech Inc, San Diego, CA) Contents are as follows:

Anti-Skeletal Alkaline Phosphatase Conjugate
Mouse monoclonal IgG (against sALP) with biotin in a bovine/horse protein matrix containing 0.1% sodium azide as a preservative. Store at 4-8 ^{O}C. This solution is stable until manufacturer expiration date on the bottle.

Streptavidin Coated Microplates

Streptavidin coated plastic well strips in a plastic frame with silica gel desiccant. Store at 4-8 ^{o}C. This solution is stable until manufacturer expiration date on the bottle.

Zero Diluent/Calibrator (A):
A bovine protein matrix containing no detectable concentration of sALP (0 ∗g sALP/L) and 0.1% sodium azide as a preservative. Store at 4-8 ^{o}C. This solution is stable until manufacturer expiration date on the bottle.

Skeletal Alkaline Phosphatase Calibrators (B-F)
A bovine protein matrix containing approximately 7, 15, 30, 60, and 90 ∗g human sALP/L, a blue dye and 0.1% sodium azide as a preservative. Refer to insert label for assigned values. Store at 4-8 ^{o}C. These solutions are stable until manufacturer expiration date on the bottles

Low Control Skeletal Alkaline Phosphatase (1)
Contains approximately 11 ∗g sALP/L in bovine protein matrix, a blue dye and 0.1% sodium azide as a preservative. Refer to insert label for assigned range. Store at 4-8 ^{o}C. This solution is stable until manufacturer expiration date on the bottle.

High Control Skeletal Alkaline Phosphatase (2)
Contains approximately 45 ∗g sALP/L in bovine protein matrix, a blue dye and 0.1% sodium azide as a preservative. Refer to insert label for assigned range. Store at 4-8 ^{o}C. This solution is stable until manufacturer expiration date on the bottle.

Wash Concentrate (A)
Detergent solution (6% Tween) containing 0.3% sodium azide as a preservative. Store at 4-8 ^{o}C. This solution is stable until manufacturer expiration date on the bottle.

(2) LIQUID SUBSTRATE/QUENCH SET (Provided separately)
Liquid Substrate/Quench Set, cat no. #4608 (Hybritech Inc, San Diego, CA)
Contents are as follows:

Substrate Concentrate
p-Nitrophenyl phosphate in a stabilizing buffer containing 0.1% sodium azide as preservatives. Store at 4-8 ^{o}C. This solution is stable until manufacturer expiration date on the bottle.

Substrate Diluent
Buffer containing preservatives. Store at 4-8 ^{o}C. This solution is stable until manufacturer expiration date on the bottle.

Quench Reagent
1 N Sodium Hydroxide Store at 4-8 ^{o}C. This solution is stable until manufacturer expiration date on the bottle

(3) Gibco normal human serum (NHS) (Gibco, Gaithersburg, MD).

(4) Distilled water (University of Washington Medical Center, Seattle, WA).

B. Reagent Preparation

Kit reagents are provided in two separate reagent sets. The antibody set provides the microtiter plate strips and frames, immunochemicals, calibrators, and controls required to form the solid phase/capture antibody/sALP complex and calibrate the assay up to 90 ∗g sALP/L. The Liquid substrate/quench set provides the reagents for the development of a color change measurable in a microtiter plate reader at 405 nm and provides quench reagent to stop substrate turnover. Kit reagents are to be stored between 2 °C and 8°C at which temperatures they are stable until the expiration date printed on the box label. (Hybritech Incorporated, San Diego, CA)

(1) Bring all reagents to room temperature (18°C – 25°C) prior to use.

(2) Thoroughly mix reagents before each use by gentle agitation or swirling.

(3) Wash Solution: To prepare wash solution, add wash concentrate to 500 mL of distilled water and mix.

(4) Substrate Reagent: To prepare substrate reagent, dilute substrate concentrate by adding 1 ml into 1 vial of substrate diluent (30ml). Alternatively, to prepare smaller volumes, dilute 1 volume of substrate concentrate into 30 volumes of diluent.

(5) Tandem∗- MP OSTASE reagents are stored between 2°C and 8°C, at which temperatures they are stable until the expiration date printed on the box label.

(6) The substrate concentrate as well as the combined substrate reagent are yellow. Combined substrate reagent is stable for 7 days when stored in the dark between 2°C and 8°C. The combined substrate reagent must not be used if the absorbance at 405 nm is greater than 0.5 units.

(7) Wash and quench reagents are stable at 2°C to 30°C until the expiration dates printed on the bottle labels.

(8) If reagent solution is turbid or contains precipitates, contact Hybritech Technical Support {EUROPE: 32 41 677000; U.S.A.: 1-800-854-1957}.

(9) All reagents must be brought to room temperature (18°C to 25°C) prior to use. After use all reagents except the wash solution should be stored at 2°C to 8°C.

(10) Unused well strips should be returned to the plastic storage tray with the desiccant pouch provided and stored at 2°C to 8°C.

C. Instrumentation

(1) Bio Tek EL 808 Automated Microtiter Plate Reader - is a 8 channel, automated, benchtop, general purpose, enzyme immunoassay analyzer

which measures the optical density of solutions in a 96-well microtiter plate at 405nm, 414nm, 450nm, 550nm, or 650nm. Data handling and reduction is performed using Bio Tek KC4 KinetiCalc for Windows. This software is a general data reduction package used to analyze data generated from colormetric microtiter plate assays as read on the Bio Tek EL808. (Bio Tek Instruments, Winooski, Vermont) This instrument includes a Hewlett Packard Laser Jet 6MP printer (Hewlett Packard, Boise ID).

OR

(1) Rosys Plato 3301 is fully automated, benchtop, general purpose, enzyme immunoassay analyzer which performs all sample, dilution, plate rotation,, and reagent handling steps including movement of plates, mixing of samples, incubation, washing and measuring the optical density of solutions in a 96-well microtiter plate at 405nm, 414nm, 450nm, 550nm, or 650nm. Data handling and reduction is performed using resident Rosys Plato 3301 software (Rosys Anthos Instruments, New Castle, DE) This instrument includes a Hewlett Packard Laser Jet 6MP printer (Hewlett Packard, Boise ID).

(2) Computer (Dell Computer Systems, Round Rock, TX).

(3) Horizontal microplate rotator (500-900 rpm range) (Labline Plate Shaker, Melrose Park, IL)

(4) Transfer pipets, cat. no. #7524 (Becton-Dickinson, Franklin Lakes, NJ).

(5) Disposable tip precision pipettors: fixed volume or adjustable for 50, 100, and 150 ∗L (±1%) Optionally, a multichannel pipette can be used together with disposable V-shaped troughs for addition of anti-sALP assay conjugate, substrate and quench reagents. (Any vendor)

(6) Repeating pipettors and disposable tips for 50, 100 and 300 ∗L (Oxford Labware, San Francisco, CA).

(7) Test tubes for sample dilutions (Any vendor**)**

(8) Microplate washer and Aspiration device (Nunc ImmunoWash 8, Cambridge, MA)

(9) Timer, minimum range of 5-90 minutes in 1 minute intervals (Any vendor)

(10) Container for storage of Wash Solution, 1 liter (Any vendor)

(11) Container for preparing Substrate Reagent, 1 liter (Any vendor)

D. Standards/Calibrator Preparation

Calibrators, Zero Diluent/Calibrator (A) and Skeletal Alkaline Phosphatase Calibrators (B-F) are received in a liquid ready to use format. No further preparation is required prior to use other than bringing to room temperature (18 ^{o}C -25 ^{o}C).

E. Preparation of Quality Control Materials

The Immunology Division prepares two levels of control from normal and/or pooled patient serum. Both pools are analyzed with each assay. Prepare in sufficient quantity to provide serum samples for 2 years. Prior to aliquoting and defining, test the stock once for approximate value and adjust if necessary. For the normal control, use normal human serum. (Gibco, Gaithersburg, MD) Mix well prior to aliquoting. For the high control, dilute normal human serum with an elevated patient serum.

Analyze newly prepared control material for at least 20 runs in parallel with the current control to determine acceptance ranges. Acceptance ranges must be determined prior to using control material for any patient run evaluations. Prepare in sufficient quantity to provide control material for at least 2 years.

Divide the stock control material into 10-mL tubes containing a volume for a 3-4 month supply and label with 'I #' and freeze at -85 ^{o}C. As needed, thaw a stock control tube and divide into approximately 150 uL aliquots to be stored for a maximum of 3-4 months at -70 ^{o}C. Thaw and use one aliquot of control material for each run.

(1) As new stock control is prepared, define a new control range by assigning the first value observed as the mean and assigning a large standard deviation. Append TEMP to the control lot number name. Prepare new blank Levey Jennings table using this temporary limits.

(2) After 20 parallel runs, use the data from the Levey-Jennings chart to assign a permanent mean and standard deviation. Normal acceptance ranges are determined as mean $\pm$ 2 standard deviations.

(3) Stock control material is aliquoted into individual use bullets. The aliquot bullet label should include the date of preparation and a letter indicating sequential aliquot. (Examples: 9/90-A for the first time this control is aliquoted, 9/90-B for the second time. Record the label on the quality control material record sheet.

(4) The lot name should include an identifying name, the date the control was prepared (month and year), and information about the control range (temp or date of calculation or recalculation).

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Calibration Curve

Calibration materials are run with each patient run. Each run is evaluated using run specific observations. See following for example of observed absorbances and curve construction.

	Description	Abs. 405 nm	Mean Abs	SALP ug/L
1	Calibrator A	0.193		
2		0.187	0.190	0.0
3	Calibrator B	0.401		
4		0.595	0.402	7.2

5 **6**	**Calibrator C**	**0.595** **0.572**	**0.584**	**15.5**
7 **8**	**Calibrator D**	**0.908** **0.868**	**0.887**	**31.9**
9 **10**	**Calibrator E**	**1.498** **1.460**	**1.479**	**58.4**
11 **12**	**Calibrator F**	**2.000** **2.051**	**2.026**	**85.8**
13	**Patient Sample**	**0.911**		**33.0**

B. Verification

The instruments used to read assay results are equipped to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the quality control manual, the entire series is invalidated.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

(1)The procedure for this assay is performed at room temperature. Bring all serum specimens and kit components to room temperature (18^{o}C to 25^{o}C) and mix well before use.

(2) All calibrators and controls should be tested at the same time and run in duplicate. Because the termination of each incubation stops a reaction that is in progress (i.e., antibody binding or substrate turnover), reliable calibration of the assay depends on ensuring that the incubation times are essentially the same for all wells.

B. Assay procedure

(1) Label test well strips and holders appropriately and load strips into holder frame. Run in duplicate each of 6 calibrators, low and high controls, and patient specimens for each assay.

(2) Pipette 50 uL of zero diluent/calibrator/controls and patient specimens into each well as labeled.

(3) Pipette 100 uL of sALP assay conjugate into each well.

(4) Incubate for 1 hour at room temperature (18^{o}C to 25^{o}C) using a horizontal rotator set at 500-900 rpm.

(5) Wash the microplate wells 3 times by:
   a. Aspirating the from the first strip.
   b. Pipetting 300uL of wash solution into the first strip.
   c. Repeating steps a) and b) on all subsequent strips.
   d. Repeating steps a) and c) twice.

(6) Dispense 150uL of substrate reagent into each well. Proceed to the next step without delay.

(7) Incubate for 13-15 minutes at room temperature (18^{o}C to 25^{o}C) using a horizontal rotator set at 500-900 rpm. Proceed to next step without delay.

(8) Dispense 100 uL of quench reagent into each well.

(9) Read the absorbance of each well at 405 nm in a microplate reader, subtracting a blank reading at 600-650 nm for each well. Read the plate within 1 hour of quenching the reaction.

(10) Calculate the results as described in Calculations.

C. Calculations

Results may be calculated by using computer-assisted methods or manually on linear graph paper.

Computer-Assisted Method

Computer assisted data reduction may be used to calculate results for the Tandem-MP Ostase Assay. A Four parameter curve fitting routine, provides acceptable results.

The Tandem-MP Ostase calibration curves generated at 405 nm may be constructed manually on liner graph paper as shown below by plotting the mean absorbance for each Calibrator on the y-axis versus the concentration of sALP on the x-axis. A point-to-point curve should be drawn through the calibration points. Do not force the curve to a straight line.

To determine the concentration of sALP in the Controls and patient specimens, extend a horizontal line from the absorbance value for the test sample to the calibration curve. At the point of intersection of the horizontal line and the curve, drop a vertical line to the x-axis and read the concentration of sALP ∗g/L.

If the absorbance of the sample is greater than the linear range of the microplate reader at 405 nm, the result must be reported as "greater than that value which corresponds to the linearity of the microplate reader," or the specimen must be diluted and re-assayed. The observed concentration of the diluted patient specimen must be multiplied by the dilution factor.

D. Recording of Data

(1) Analytical Results Data
KC4 software (or Rosys Plato 3301) automatically calculate and print analytical results as derived from run specific calibration data. Any repeat measurements, special dilutions, or results exceeding the measuring range of a determination are repeated at appropriate dilutions. Results are corrected by that dilution prior to reporting. Example: a sample diluted 1:5 is observed to have an uncorrected value of 35.3 ug sALP/L. Final result is reported as 176.5 ug sALP/L.

(35.3 ug sALP/L x 5 = 176.5 ug sALP/L)

Specimen results are entered into the assay specific results table created from the send file corresponding to the specific sample box using Excel software (Microsoft Corporation, Redmond WA).

(2) Quality Control Data

Control results are entered into the assay specific Levey Jennings table and plot if they are found to be in compliance with Westgard rules The evaluated copy of the table is printed out and checked for accuracy of data entry.

E. Procedural Notes

(1) Note on Plate Washing: Immunometric assays require efficient washing to remove the unbound labeled antibody. Therefore, it is very important to wash each well efficiently, removing the last droplets of the Wash Solution to achieve optimal results.

(2) If a specimen was found to contain greater than sALP at a concentration greater than the highest calibrator, the specimen should be diluted with the Zero Diluent/Calibrator and should be reassayed according to the assay procedure. The dilution factor must be incorporated into the calculation of results. Each diluted specimen should be mixed thoroughly prior to testing. The recommended dilutions for specimens containing greater sALP greater than the highest calibrator are 1:3, 1:5, or 1:10. However, it is desirable to dilute the serum specimens with sALP greater than the highest calibrator so that the diluted sample reads greater than 10 ug sALP/L.

(3) Because absorbance is a function of temperature and duration of the Substrate Reagent incubation, it is very important that this incubation be the same for all wells/plate. This can be accomplished by ensuring that the elapsed time for pipetting reagents from beginning to end (without interruption) is exactly the same for both the Substrate Reagent addition step and the Quench Reagent addition step. To ensure best results, the addition of these reagents should not exceed 90 seconds and total substrate incubation should not exceed 15 minutes.

(4) For convenience, repeating or multichannel pipettors may be used for dispensing assay conjugate, wash solution, substrate and quench reagent. Pipettors with disposable tips are recommended for pipetting calibrators, controls and specimens. The pipette tips should be changed after each sample is pipetted to avoid potential sample carryover and contamination of the reagents or specimens.

(5) Avoid microbial contamination of the reagents when removing aliquots from the vials

(6) Do not mix materials from different kit lots

(7) Do not use kit components beyond the expiration date

9. REPORTABLE RANGE OF RESULTS

Report values to the nearest 0.1 ug/L. Reportable range is from 0.7 ug sALP/L to approximately 80 ug sALP/L . The upper reportable value is determined by the calibration material supplied with the kit from the manufacturer. Values exceeding this upper limit are repeated on dilution on a following run until uncorrected values fall between 7 ug sALP/L to approximately 80 ug sALP/L. Report values <0.7 as <0.7 ug sALP/L.

10. QUALITY CONTROL (QC) PROCEDURES

A. Good laboratory practices include the use of control specimens within an assay run to ensure that all reagents and protocols are performing properly.

B. Recovery of control concentration should fall within the stated range. If the controls are out of range:

- Verify that the microplate reader is correctly programmed.
- Verify that the controls have not exceeded the expiration date.
- Check the control ranges for accuracy.
- Verify that the order of addition has not been changed.

C. The coefficient of variation (%CV) of the 405 nm absorbance readings for each calibrator and control sample should be less than 10%.

D. All micro-pipettors used in testing clinical specimens should be checked for calibration every 3 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

E. Estimates of imprecision can be generated from long-term quality control pool results. Bench quality controls are used in this analytical method. Bench quality control specimens are inserted by the analyst at least once in each analytical run (a set of consecutive assays performed without interruption) so that judgments may be made on the day of analysis. The data from these materials are then used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

F. The bench controls are prepared in sufficient quantity to provide serum samples for all the assays for 2 years. Ranges are established after 20 parallel runs with previously established controls. Ranges are established by using the formulas for statistical calculations data. The quality control pools comprise two levels of concentration spanning the low and high ranges bone alkaline phosphatase.

G. Standards and bench quality controls are placed at the beginning of each analytical run. After analysis, the long-term quality control charts (Levey-Jennings) for each control material are consulted to determine if the system is "in control." The Levey Jennings chart plots the means of the duplicate determinations on the y axis and the date of the observation on the x-axis. Quality control material observations are compared with the 95% and 99% confidence limits as well as with the center line (the overall mean of the characterization runs) prior to reporting any results. The system is out of control if any of the following events occur for any one of the quality control materials:

- The mean from a single pool falls outside the 99% confidence limits.
- The means from two pools fall either both above or both below the 95% confidence limits.
- The means from eight successive runs for one pool fall either all above or all below the center-line.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the run is declared "out of control", the system (instrument, calibration standards, reagents etc.) are investigated to determine the root of the problem before any results are released. Consult with the supervisor for appropriate actions.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

A. Report values to the nearest 0.1 ug/L. Reportable range is from 0.7 ug sALP/L to approximately 80 ug sALP/L . The upper reportable value is determined by the calibration material supplied with the kit from the manufacturer. Values exceeding this upper limit are repeated on dilution on a following run until values, prior to correction for dilution, fall between 7 ug sALP/L to approximately 80 ug sALP/L.

B. The minimum detectable concentration of sALP of this assay is less than 0.7 ∗g sALP/L. The minimum detectable concentration is defined as that concentration of sALP which corresponds to the absorbance that is two standard deviations greater than the mean absorbance of 20 replicate determinations of the zero diluent/calibrator (A).

C. HAMA Interference: Some individuals have antibodies to mouse protein (HAMA, human anti-mouse antibodies), which can cause interference in immunoassays that employ antibodies derived from mice. In particular, it has been reported that serum samples from patients who have undergone therapy or diagnostic procedures that include infusion of mouse monoclonal antibody may produce erroneous results in such assays. Therefore Tandem-MP OSTASE results for such patients should be used only in conjunction with results from some other diagnostic procedure and with information available from the clinical evaluation of the patient.

D. The immunoreactivity of liver ALP has been determined in this a: 100U/L of liver ALP activity gives a result of 2.8 to 6.2∗g/L in the this assay.. Serum samples with significant elevations of liver ALP activity may yield elevated results in this assay. Patients with metabolic bone disorders who have low levels of disease activity may have skeletal ALP levels that fall within the expected values.

E. These results should be used only in conjunction with information available from the clinical evaluation of the patient and other diagnostic procedures. Therefore, is assay is not recommended for use as a screening procedure to detect the presence of osteoporosis in the general population. Furthermore, this assay cannot be used to assess the rate of bone formation or bone remodeling.

F. The liver ALP reactivity was determined using serum samples from liver disease patients and serum samples from patients with Paget's disease. Samples were screened by electrophoresis and shown to contain >95% liver ALP or sALP. Two methods were used to evaluate liver ALP reactivity.

(1) The first procedure, used a heat inactivation method that minimizes the contribution of endogenous sALP in the samples with elevated liver ALP. Using this method 100 U/L liver ALP produced a result of 2.8 to 3.4 ∗g/L in this assay.

(2) The second procedure, used the slope method (sALP vs. total ALP), where no pretreatment of the samples occurred. In this study, quantitation of the liver ALP and sALP samples (y-axis) was plotted against the total ALP activity (x-axis) in each sample. From the slope values of the liver ALP and sALP samples, it was determined that 100 U/L of liver ALP activity produced a result of 6.2 ∗g/L in the Ostase assay; and100 U/L of sALP activity produced a result of 36.9 ∗g/L in the Ostase assay.

13. REFERENCE RANGES (NORMAL VALUES)

Male	3.7-20.9 ug/L
Premenopausal female	2.9-14.5 ug/L
Postmenopausal female	3.8-22.6 ug/L

	N	SALP Mean ug/L	SD	SALP Median ug/L	SALP 95th percentile ug/L
Males	217	12.3	4.3	11.	20.1
Pre-Menopausal females	228	8.7	2.9	8.5	14.3
Post menopausal females	529	13.2	4.7	2.5	22.4

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable to this procedure.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens should be maintained at 20-25 ^{o}C during testing. After testing, the samples are stored at <- 70^{o}C.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There are no acceptable alternative methods of analysis. Specimens may be stored at 4-8 ^{o}C for no longer than 2 days. Otherwise, specimens should be stored at $\leq$-70^{o}C until the system is returned to functionality.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable to this procedure.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Standard record keeping should be used for tracking specimens. The primary results include daily test results as well as stored quality control results.

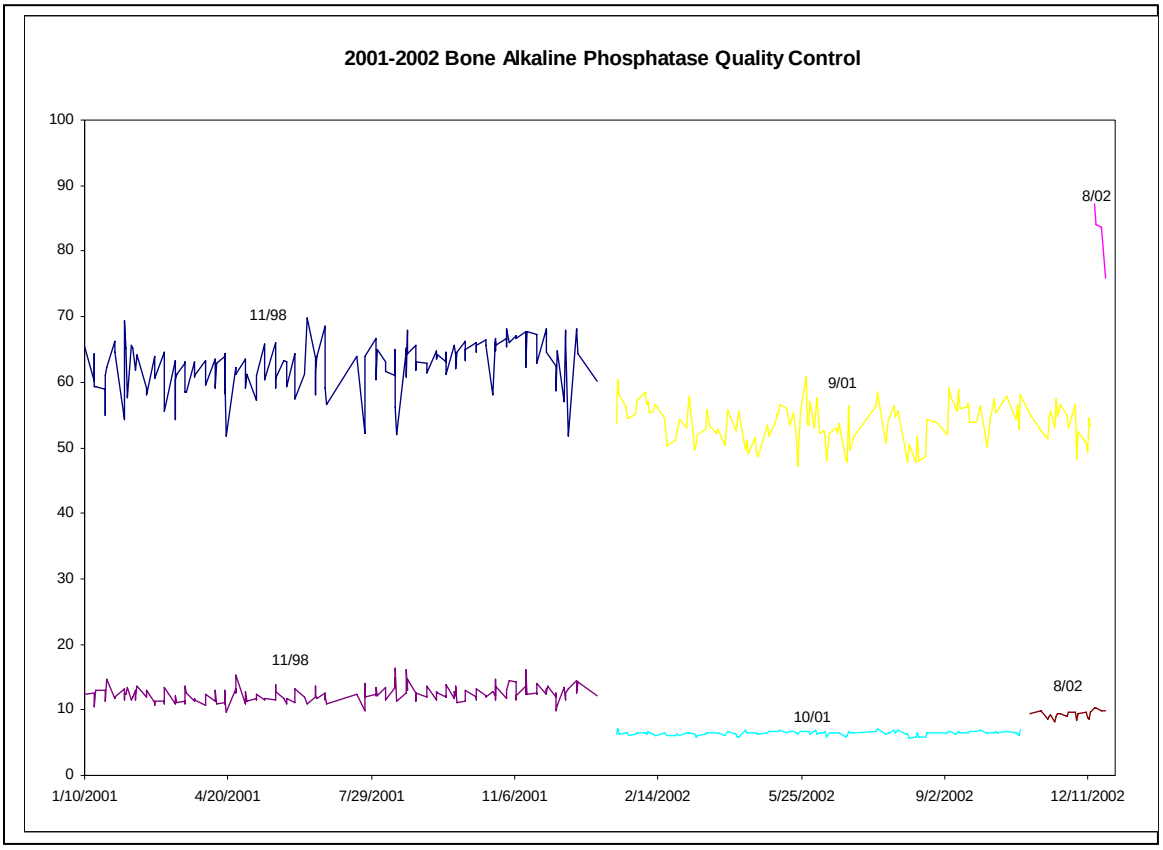
The original NHANES III ship file is copied into a template Excel file and onto the hard drive of the IBM computer. After the results are entered into the database and assay results transmitted electronically files are stored for 6 months on a server that is backed up on a daily basis. After 6 months, the result files are transferred onto a CD along copies of original ship files and QC information.

The residual serum is stored at $\leq$-70 ^{o}C for 3 months after analysis; then it is returned to the NHANES Repository in Rockville, MD for long-term storage.

19. SUMMARY STATISTICS AND QC GRAPHS

Summary Statistics for Bone Alkaline Phosphatase by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
11/98	172	1/10/2001	1/3/2002	62.7	3.57	5.7
11/98	178	1/10/2001	1/3/2002	12.3	1.13	9.1
9/01	133	1/16/2002	12/13/2002	53.8	2.96	5.5
10/01	114	1/16/2002	10/25/2002	6.5	0.30	4.7
8/02	23	11/1/2002	12/23/2002	9.3	0.56	5.9
8/02	4	12/16/2002	12/23/2002	82.7	4.85	5.9



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