Appendices
APPENDIX-1

ESTIMATION OF BONE SPECIFIC ALKALINE PHOSPHATASE (BAP)

Method: Enzyme linked immunosorbent assay

Principle: The OCETIA Ostase BAP assay is a solid phase, monoclonal antibody immunoenzymatic assay. Samples containing BAP are reacted with a solution containing a biotin-labeled, BAP-specific monoclonal antibody. The reaction takes place in plastic well strips (solid phase) coated with streptavidin and enclosed in a plastic frame. Following the formation of a solid phase/capture antibody/BAP complex, the micro plate is washed to remove unbound BAP 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 450 nm in a micro plate reader. The absorbance is proportional to the concentration of BAP present in the test sample. The calculation of BAP concentration in the sample is based on concurrent testing of BAP calibrators and zero Calibrator/Diluent.

Materials Required

1. Disposable tip precision pipettors: fixed volume or adjustable for 50, 100 and 150µl (± 1%).
2. Optionally, a multi-channel precision pipette can be used together with disposable V-shaped troughs for addition of anti-BAP assay conjugate, Substrate and Quench reagents.
3. Disposable pipette tips for 50, 100, 150µl
4. Test tubes for sample dilutions
5. Micro plate washer
6. Aspiration device
7. Distilled water
8. Timer
9. Container for storage of wash solution
10. Micro plate reader (405 nm primary wave length, 600-650 nm background subtraction) and data reduction software.
11. Horizontal micro plate rotator (500 rpm to 900 rpm range)
Preparation of reagents

1. Bring all reagents to room temperature (18-25°C) prior to use.
2. Thoroughly mix reagents before each use by gentle agitation or swirling
3. Use a clean pipette tip for each specimen, calibrator or control to avoid contamination
4. Wash solution: To prepare wash solution, add wash concentrate A to 500 ml of distilled water and mix.

Procedure

1. Label test well strips/holders appropriately and load strips into holder frame. Run in duplicate each of 6 calibrators, low and high controls for each assay.
2. Pipette 50µl of zero calibrator/calibrator B-F/calibrators/patients specimens into the bottom of each assigned well.
3. Pipette 100µl of conjugate into each well.
4. Incubate for 1 hour at 18-25 °C using a horizontal rotator set at 500 rpm to 900 rpm.
5. Wash the micro plate wells 3 times by:
   a. Aspirating the liquid from the first strip
   b. Pipetting 300µl 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 150µl of substrate reagent into each well. Proceed to next step without delay.
8. Dispense 100µl of Quench Reagent into each well.
9. Read the absorbance of each well at 405 nm in a micro plate reader, subtracting a blank reading at 600-650 nm for each well. Read the plate with in 1 hour of quenching the reaction.

Calculation of Results

Results may be calculated by using computer-assisted methods or manually on linear graph paper
STANDARD CURVE FOR THE ESTIMATION OF BONE SPECIFIC ALKALINE PHOSPHATASE
APPENDIX -II

ESTIMATION OF TARTRATE-RESISTANT ACID PHOSPHATASE-5b (TRAP-5b)

Method: Enzyme linked immunosorbent assay

Principle:

1. The plate is coated with anti-TRACP antibodies (monoclonal).
2. Calibrators, control and patient samples are added.
3. Releasing reagent is added.
4. Dissociation of active TRACP 5b from the binding proteins.
5. TRACP-5b is bound by the anti-TRACP antibodies and
6. Incubation with pNPP substrate

Reagents and materials required:

1. Water for injection (H₂O redist.). Use of deionised water can disturb the test procedure.
2. Adjustable micropipettes.
3. Clean glass or plastic containers for dilution of wash buffer and specimen.
4. Suitable device for micro plate washing (e.g. multistepper or ELISA washer).
5. Incubator for 37°C
6. Micro plate shaker, shaking frequency 850-950 rpm, amplitude 4 mm.
7. Micro plate reader with filter for 405 nm.

Preparation of the Reagents:

Before starting the test procedure all kit components must be equilibrated to room temperature.

1. Microplate

   The aluminium bag has to be tightly resealed together with the desiccant after each removal of wells.

2. Wash buffer

   Mix one volume of wash buffer (10x) with nine volumes of water for injection (e.g. 50 ml Wash Buffer (10x) with 450 ml water). Seven ml of diluted wash buffer is needed for 8 wells
3. **Calibrators**

Reconstitute the lyophilized calibrators each with 0.5 ml of water for injection. Reconstitution time 15 min.

4. **Control**

Reconstitute the lyophilized control with 0.5 ml of water for injection. Reconstitution time 15 min.

5. **Substrate Solution**

One substrate Tablet is dissolved in 5 ml Substrate Buffer. The substrate solution must not be stored.

**Procedure**

1. Cut the aluminum bag above the zip fastener and take out the required number of the micro plate wells
2. Add 100μl sample Diluent as blank to the first wells of the plate. To the next wells, add 100μl each of calibrators, control and samples in duplicates
3. Add 50μl Releasing Reagent to each well.
4. Seal the micro plate with incubation cover foil and incubate for 60 min (± 5 min) at the room temperature with constant shaking at 850-950 rpm.
5. After incubation wash the micro plate wells four times with 300μl wash buffer per well. Pay attention that all wells are filled. After washing tap micro plate wells on filter paper.
6. Add 100μl substrate solution to each well.
7. Seal the micro plate with incubation cover foil and incubate for 60 min (± 5 min) at 37°C (± 1°C)
8. Stop the reaction by adding 25μl of Stop Solution to each well.

The reading should be done within 15 min after adding the stop solution.

**Calculation of Results**

1. Read OD values at 405 nm.
2. Subtract the average OD value of the blank from all other OD values.
3. The average OD values of the calibrators are plotted against the activity values.
STANDARD CURVE FOR THE ESTIMATION OF TARTRATE RESISTANT ACID PHOSPHATASE-5b
APPENDIX -III

ESTIMATION OF 25-HYDROXY VITAMIN D3 (25-OH VITAMIN D3)

Method: Radioimmunoassay

Principle: At first standards and samples (serum or plasma) are extracted with acetonitrile. A fixed amount of $^{125}$I labeled 25 OH Vitamin D3 competes with the 25 OH Vitamin D3 from either extracted samples or standards for a fixed amount of specific antibody sites immobilized to the lower and inner surface of plastic tubes. After 2 hours incubation at room temperature, an aspiration step stops the competition reaction. The tubes are then washed with 3 ml washing solution and counted in a gamma counter.

Supplies not provided: The following material is required but not provided in the kit: Distilled water, Ethanol absolute, Pipettes for delivery of: 50µl, 100µl, 200µl, 400µl, 1ml.

Glass tubes (12x75 mm) for extraction step, vortex mixer, centrifuge operating at 1500g, magnetic stirrer, 5ml automatic syringe for washing, aspiration and washing device, Gamma counter for $^{125}$I counting.

Reagent preparation:

Standards: Reconstitute the zero standards with 1 ml distilled water and other standards with 1 ml distilled water.

Controls: Reconstitute the controls with 1ml distilled water.

$I^{125}$ OH- Vit.D3: Reconstitute with 6 ml of a mix of distilled water/ethanol (50/50).

Wash solution: Dilute the content of the water buffer in 700 ml distilled water; use a magnetic stirrer to homogenize.

Procedure:

Extraction step:

1. Label glass tubes (12x75 mm) for extraction: 6 standards, 2 controls and up to 40 samples.

2. Add 0.5 ml acetonitrile to each tube.

3. Dispense 200µl of each standard, control or sample in the respective tubes.
4. Mix for 7 seconds with a vortex.

5. Centrifuge for 5 minutes at room temperature (at 800 g).

**Incubation step:**

1. Label coated tubes, in duplicate, for each standard, control and sample. For determination of total counts, label 2 normal tubes.

2. Add 100μl of the supernatant obtained after the extraction step in the corresponding tubes. Pipette tips have to be saturated with corresponding supernatant before the addition in the tube.

3. Dispense 400μl incubation buffer in each tube, except those for total counts.

4. Add 50μl tracer in each tube, including total counts.

5. Shake the tube rack gently.

6. Incubate for 2 hours, at room temperature.

7. Aspirate the content of each tube (except total counts).

8. Wash tubes twice with 2 ml Wash solution and aspirate. Avoid foaming during the addition of the wash solution. After the washing, let the tubes standing upright for two minutes and aspirate the remaining drop of liquid.

9. Count the tubes in a gamma counter for 60 seconds.

**Calculation of results:**

1. Calculate the mean of duplicate determinations, rejecting obvious outliers.

2. Calculate the bound radioactivity as a percentage of the binding determined at the zero standards point (0) according to the following formula:

\[
\text{Counts (Calibrator or sample)} \times 100
\]

\[
\text{Counts (Zero Calibrator)}
\]

\[
\frac{\text{Counts (Calibrator or sample)}}{\text{Counts (Zero Calibrator)}} = \frac{\text{B}}{\text{B}_0} \times 100
\]

3. Using a 3 cycle semi-logarithmic or logit-log graph paper plot the \((\text{B}/\text{B}_0 \times 100)\) values for each standard point as a function of the 25 OH.D3 concentration of each standard point. Computer assisted method can also be used to construct the calibration curve.

4. By interpolation of the samples \((\text{B}/\text{B}_0 \times 100)\) values, determine the 25 OH.D3 concentrations of the samples from the reference curve.
5. For each assay, the percentage of total tracer bound in the absence of unlabelled 25 OH.D3 (B0/T) must be checked.

Intra assay variation: - 7.1

Inter assay variation: - 8.3

Recovery: - 97%
STANDARD CURVE FOR THE ESTIMATION OF 25(OH)VITAMIN D3
APPENDIX-IV

ESTIMATION OF LIPID PEROXIDATION PRODUCTS - SERUM MALONDIALDEHYDE

Method: Serum Malondialdehyde was estimated by the method of Okhawa et al., 1974. The most frequently used to study lipid peroxidation is the measurement of thiobarbituric acid (TBA) reactive substances.

Principle: The lipids in the cell membranes are highly susceptible to per oxidative damage and are broken down into number of units to form malondialdehyde. This reacts with thiobarbituric acid (TBA) to form thiobarbituric acid reacting substance (TBARS), which has a pink colour with absorption maximum at 532 nm. A molecule of malondialdehyde reacts with two molecules of thiobarbituric acid (TBA) to result a Schiff base product. Thiobarbituric acid was added to samples under acidic condition and the absorption of the colour, which develops after heating, was estimated at 535 nm.

Reagents

1. 20% trichloro acetic acid
2. TBA reagent (200 mg of thiobarbituric acid in 30 ml distilled water and 30 ml of acetic acid).
3. N- butanol
4. Normal Saline (0.9% NaCl)

Standard

1,1,3,3 Tetramethoxy propane.

Stock Standard

0.1ml or 100μl of concentrated Tetramethoxy propane was taken and made up to 10.1 ml with ethanol.

Working Standard

0.1 ml or 100μl of stock standard solution was taken and made up to 10.1 ml with ethanol from that different dilutions were made for different concentrations.
Procedure:

To each test tube 0.5 ml of serum, 0.5 ml of Normal saline, 1 ml of 20% trichloro acetic acid (TCA) and 0.25 ml of TBA reagent were added. The tubes were kept in boiling at 95°C for 1 hour. To the contents of the tubes, 3 ml of n-butanol was added, mixed well for 15 minutes. After extraction the tubes were centrifuged at 3000 rpm for 10 minutes and the separated butanol layer was collected and read against a reagent blank at 535 nm.

Lipid peroxidation is expressed in terms of nano moles of malondialdehyde (MDA) per ml of plasma or serum.
STANDARD CURVE FOR THE ESTIMATION OF LIPID PEROXIDATION PRODUCTS - MALONDIALDEHYDE
APPENDIX -V

ANTIOXIDANT ENZYME ACTIVITIES

Measurement of Erythrocyte catalase activity:

Erythrocyte catalase activity was assayed by the method of Aebi.

Preparation of hemolysate

The erythrocytes after the removal of plasma and leukocytes were washed three times with isotonic sodium chloride (0.9% NaCl). A stock hemolysate containing ~5gmHb/100 ml was prepared by lysing the RBC with cold distilled water. A 1:500 dilution of this concentrated hemolysate was prepared with phosphate buffer immediately before the assay was performed and hemoglobin content in the hemolysate was determined by Drabkins method.

Reagents

1. Phosphate buffer (50 mM) pH 7.0
2. Hydrogen peroxide (30 mM): 340μl of 30% H₂O₂ was dissolved in 100 ml of phosphate buffer.

Two ml each of hemolysate was taken in sample cuvette and reference cuvette (blank) and added 1 ml of 30 mM H₂O₂ to sample cuvette and 1 ml of phosphate buffer to reference cuvette and the decrease in the absorbance was recorded for 15 sec. at 240 nm in a spectrophotometer.

Hemoglobin in Hemolysate

The hemoglobin content in hemolysate was measured by the method of Drabkin and Austin, 1932.

Principle

Hemoglobin is oxidized to methemoglobin, which combines with cyanide to form cyanomethemoglobin. The absorbance of the solution is then measured at 540 nm.

Reagents

1. Drabkins Reagent: This reagent was obtained commercially.
2. Cyanomethaemoglobin: this was obtained commercially and had a Hb concentration of 16 g/dl.
Procedure

0.02 ml of hemolysate was diluted with 5.0 ml of Drabkins reagent. The diluted solution was mixed well and allowed to stand for 10 min. at room temperature to ensure completion of the reaction. The solution was read at 540 nm together with the standard solution of cyanomethaemoglobin. The reagent blank was used to set the optical density to zero.

Calculation

The catalase activity was calculated by using the formula given below and was expressed in KU/gm Hb.

\[ \text{KU/gm Hb} = \frac{k}{\text{ml}} \left( \frac{1000}{b} \right) - \frac{2.315}{15} \left( \frac{a}{b} \right) (\log A_1/\log A_2) \text{ sec}^{-1} \]

Here \( A_1 \) is \( A_{240} \) at \( t = 0 \), \( A_2 \) is \( A_{240} \) at \( t = 15 \) sec. \( a \) is the dilution factor (Hb concentration in blood or erythrocyte sediment (mg Hb/ml) / Hb concentration in cuvette (mg Hb/ ml) and \( b \) is the Hb content of blood or erythrocyte sediment (grams/lit).
APPENDIX - VI

ESTIMATION OF GLUTATHIONE-S-TRANSFERASE ACTIVITY

**Method:** Glutathione-S-Transferase activity was estimated by the method of Habig et al., 1974.

**Principle**

Glutathione-S-transferase activity was measured by following the increase in absorbance at 340 nm, using 1-chloro-2, 4-dinitrobenzene as the substrate.

**Reagents**

1. 0.3 M phosphate buffer, pH 6.5
2. 30 mM Glutathione
3. 30 mM 1-chloro-2,4-dinitrobenzene (CDNB prepared in 95% alcohol).

**Procedure**

The following solutions were taken in a cuvette in the given proportion.

To each test tube, 1ml of phosphate buffer, 0.1 ml CDNB and 0.1 ml serum were added. The volume was adjusted to 2.9 ml with distilled water. The reaction mixture was incubated at 37°C for 5 minutes and the reaction was started by the addition of 0.1 ml of 30 mM glutathione. The absorbance was followed for 5 min at 340 nm. Reaction mixture without the serum was used as the blank.
APPENDIX-VII

ESTIMATION OF C-TERMINAL TELopePTIDES OF TYPE-I COLLAGEN (CTX)

Method: Enzyme linked immunosorbent assay

Principle: The urine cross laps ELISA assay is based on the competitive binding of the anti-cross laps antibodies to either soluble cross laps antigen or to cross laps antigen-coated micro-titer wells. Standards, controls, or unknown samples are pipetted into the appropriate micro titer wells. Then anti- crosslaps antibodies are added and incubation takes place for 1 hour at 18-22°C. The wells are washed and peroxidase conjugated anti-rabbit immunoglobulin is added. The wells are then incubated again for 1 hour at 18-22°C. After a second washing step the wells are incubated for 15 min with a chromogenic substrate. The reaction is stopped and the absorbance is measured.

Materials required

1. Container for preparing the washing solution
2. Precision micropipettes to deliver 15μl
3. Distilled water
4. Precision 8-multipipette to deliver 100μl
5. Micro well mixing apparatus (300 rpm)
6. Microtiter plate reader

Procedure

1. Pipette standards or samples

   Pipette 150μl of either standards (vial A-F), Control (Vial Co) or unknown samples into appropriate wells.

2. Incubation in immunostrips

   Add 100μl Primary antibody Solution (vial no .1) to each well. Cover the immunostrips with sealing tape and incubate for 60 min ± 5 minutes at 18-22°C on a microtiter plate mixing apparatus (300rpm).
3. **Washing**

   Wash the immuno strips 5 times manually with diluted Washing Solution, diluted 1:50 in distilled water.

   Usually 3-5 washing cycles are adequate. Make sure that the wells are completely emptied after each manual or automatic washing cycle.

4. **Incubation with peroxidase Conjugated Antibody**

   Add 100μl of peroxidase Conjugated Antibody to each well, cover with sealing tape, and incubate for 60 mins ± 5 minutes at 18-22°C on the mixing apparatus (300 rpm).

5. **Washing**

   Proceed as described in step 3.

6. **Incubation with chromogenic substrate solution**

   Pipette 100μl of the substrate solution into each well, cover with sealing tape, and incubate for 15 mins ± 2 minutes at 18-22°C in the dark on the mixing apparatus (300 rpm).

7. **Stopping of color reaction**

   Pipette 100μl of stopping solution into each well.

8. **Measurement of absorbance**

   Measure the absorbance at 450 nm with 650 nm as reference with in two hours.

**Calculation of Results**

Calculate the mean of the duplicate absorbance determinations. Construct a standard curve on log-linear graph paper by plotting the mean absorbance’s of the six standards A-F (ordinate) against the corresponding crosslaps concentrations (abscissa). Draw the best fitting curve. Alternatively, a four parametric logistic curve fit can be used.

Determine the cross laps concentrations of the control (co) and each patient samples by interpolation.
Calculation of corrected cross-laps value:

For each sample the cross laps concentration (μg/l) and the creatinine concentration (mM = mmol/L) should be determined. For determination of creatinine the method is Jaffe's method or equivalent is recommended.

The following equation corrects the cross-laps concentrations for variation in urine concentration:

\[
\text{Corr. CrossLaps Value (μg/mmol)} = \frac{\text{Cross laps (μg/l)}}{\text{Creatinine (mM)}}
\]
STANDARD CURVE FOR THE ESTIMATION OF C-TERMINAL TELOPEPTIDES OF TYPE-I COLLAGEN
APPENDIX-VIII

Logistic Regression

Case Processing Summary

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a. If weight is in effect, see classification table for the total number of cases.

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Block 0: Beginning Block

Classification Table* 3

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a. Constant is included in the model.
b. The cut value is .500

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Block 1: Method = Backward Stepwise (Conditional)

Omnibus Tests of Model Coefficients

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165
### Model Summary

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### Classification Table

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*a. The cut value is .500*

### Variables in the Equation

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*a. Variable(s) entered on step 1: SERCA, SERPHO, TALP.*

### Model If Term Removed

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*a. Based on conditional parameter estimates*