



Osteocalcin (Intact) ELISA

For the quantitative determination of intact osteocalcin in serum

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number:	38-OSTHU-E01
Size:	96 wells
Version:	090504/1 - ALPCO 2/4/2010

ALPCO Diagnostics

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I. INTENDED USE

This kit is an immunoenzymatic assay for the quantitative measurement of intact human osteocalcin (OST) in serum. This kit is for research use only. It is not for use in diagnostic procedures.

II. BACKGROUND

a. Biological activities

Osteocalcin or bone Gla protein (BGP) is the major non-collagen protein of the bone matrix. It has a molecular weight of 5,800 Da and contains 49 amino-acids, including 3 residues of gamma-carboxyglutamic acid. Osteocalcin is synthesized in the bone by the osteoblasts. After production, it is partly incorporated in the bone matrix and the rest is found in the blood circulation. The exact physiological function of osteocalcin is still unclear. A large number of studies show that the circulating levels of osteocalcin reflect the rate of bone formation.

b. Research applications

The determination of blood levels of osteocalcin is valuable for the study of:

- Risk of osteoporosis in women
- Bone metabolism:
 - during perimenopause and postmenopause
 - during hormone replacement therapy and treatment of premenopausal women with LH-RH (Luteinizing hormone-releasing hormone) agonists
 - in individuals with growth hormone deficiency, hypothyroidism, hyperthyroidism, and chronic renal failure

III. PRINCIPLES OF THE METHOD

The Osteocalcin ELISA is a solid phase Enzyme Linked ImmunoSorbent Assay performed on breakable microplates. The assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of human osteocalcin. Calibrators and samples react with the capture monoclonal antibody (Mab 1) coated on the microplate wells and with a monoclonal antibody (Mab 2) labeled with horseradish peroxidase (HRP). After an incubation period that allows the formation of a sandwich: coated Mab 1 – human osteocalcin – Mab 2 – HRP, the microplate is washed to remove unbound enzyme labeled antibody. Bound enzyme-labeled antibody is measured through a chromogenic reaction. Chromogen solution (TMB, ready for use) is added and incubated. The reaction is stopped with the addition of stop solution and the microplate is then read at the appropriate wavelength. The amount of substrate turnover is determined colorimetrically by measuring the absorbance, which is proportional to the osteocalcin concentration.

A calibration curve is plotted and the OST concentration in samples is determined by interpolation from the calibration curve.

IV. REAGENTS PROVIDED

Reagents	96 tests Kit	Color Code	Reconstitution
Microplate: 96 anti-OST (monoclonal antibodies) coated breakable wells	96 wells	blue	Ready for use
Conjugate Buffer: TRIS-HCl buffer with bovine serum albumin, bovine casein, EDTA, gentamycin, and thymol	1 vial 12 ml	red	Ready for use
HRP Conjugate Concentrate: HRP labeled anti-OST (monoclonal antibodies) in stabilizing buffer, 50X	1 vial 0.4 ml	red	Dilute 1:50 with conjugate buffer
Zero Calibrator: human serum with protease inhibitors and benzamidine	1 vial lyophilized	yellow	Add 1.0 ml deionized water
Calibrator N = 1 to 5: (see exact values on vial labels) in human serum with protease inhibitors and benzamidine	5 vials lyophilized	yellow	Add 0.5 ml deionized water
Wash Solution Concentrate: Tris-HCl, 200X	1 vial 10 ml	brown	Dilute 1:200 with deionized water (use a magnetic stirrer).
Controls N = 1 or 2: human serum with protease inhibitors, benzamidine, and thymol	2 vials lyophilized	silver	Add 0.5 ml deionized water
Chromogen: Chromogenic TMB (Tetramethylbenzidine) solution	1 vial 12 ml	black	Ready for use
Stop Solution: 1 N HCl	1 vial 12 ml	white	Ready for use

- Note:** 1. Use the zero calibrator for sample dilutions.
2. The OST calibrator is calibrated on a synthetic peptide (Peninsula 6045).

V. SUPPLIES NOT PROVIDED

The following material is required but not provided in the kit:

1. High quality deionized or distilled water
2. Trasyolol at 10,000 IU/ml
3. Pipettes for delivery of: 25 μ l, 100 μ l, 200 μ l, 500 μ l, and 1 ml (the use of accurate pipettes with disposable plastic tips is recommended)
4. Vortex mixer (reagent prep) and refrigerated centrifuge (recommended, sample prep)
5. Magnetic stirrer
6. Washer for Microplates
7. Microplate reader - 450 nm (reference filters 650 or 630 nm) (bichromatic reading)

VI. REAGENT PREPARATION

- A. Calibrators:** Reconstitute the zero calibrator with 1.0 ml of deionized water and the other calibrators with 0.5 ml of deionized water.
- B. Controls:** Reconstitute the controls with 0.5 ml of deionized water.
- C. Working HRP Conjugate:** Prepare an adequate volume of working conjugate solution by adding 40 μ l of the HRP conjugate concentrate to 2 ml of conjugate buffer. Use a vortex to homogenize. Extemporaneous preparation is recommended.

- D. Working Wash Solution:** Prepare an adequate volume of working wash solution by adding 199 volumes of deionized water to 1 volume of wash solution concentrate (1:200). Use a magnetic stirrer to homogenize. Discard any unused working wash solution.

VII. STORAGE AND EXPIRY DATING OF REAGENTS

- Before opening or reconstitution, all kits components are stable until the expiry date indicated on the vial labels if kept at 2-8°C.
- Unused strips must be stored at 2-8°C in a sealed bag containing a desiccant until the expiry date.
- After reconstitution, calibrators and controls are very unstable; use them immediately after reconstitution. For longer storage periods, aliquots should be made and kept at -20°C for a maximum of 6 weeks. Freezing should be performed immediately after use, do not wait to freeze until all the samples are pipetted. Avoid subsequent freeze-thaw cycles.
- The wash solution concentrate is stable at room temperature until the expiry date.
- Newly prepared working wash solution should be used on the same day as its preparation.
- After its first use, the HRP conjugate concentrate is stable until the expiry date if kept in the original well-closed vial at 2-8°C.
- Alterations in the physical appearance of kit reagents may indicate instability or deterioration.

VIII. SAMPLE COLLECTION AND PREPARATION

- Collect blood by venipuncture, taking care to avoid hemolysis, the samples must be kept in an ice bath. Separate the serum from the cells within 3 hours, the use of a refrigerated centrifuge is recommended. Add 100 µl of Trasylol (10,000 IU/ml) to the serum immediately after centrifugation (to obtain 1,000 IU Trasylol per ml sample).
- With this treatment the samples are stable for 3 days at 2-8°C. If there will be a longer delay, the samples have to be frozen (- 20°C); however, the samples can only be thawed once! For repeat testing, freeze the samples in aliquots and discard each sample after the first thawing.
- Do not use hemolyzed samples or lipemic samples.

IX. PROCEDURE

A. Handling notes

Do not use the kit or components beyond the expiry date.

Do not mix materials from different kit lots.

Bring all the reagents to room temperature prior to use.

Thoroughly mix all reagents and samples by gentle agitation or swirling.

Run calibrators, controls, and samples in duplicate. Vertical alignment is recommended. Use a clean plastic container to prepare the working wash solution.

In order to avoid cross-contamination, use a clean disposable pipette tip for the addition of each reagent and sample.

When dispensing the chromogen solution and the stop solution, avoid pipettes with metal parts.

High precision pipettes or automated pipetting equipment will improve the precision. Respect the incubation times.

To avoid drift, the time between pipetting the first calibrator and the last sample must be no longer than 30 minutes.

Prepare a calibration curve for each run; do not use data from previous runs.

Dispense the chromogen solution within 15 minutes of washing the microplate.
 During incubation with the chromogen solution, avoid direct sunlight on the microplate.

B. Procedure

1. Select the required number of wells for the run. The unused wells should be resealed in the bag with a desiccant and stored at 2-8°C.
2. Secure the strips into the holding frame.
3. Pipette 25 µl of each calibrator, control, and sample into the appropriate wells.
4. Pipette 100 µl of working HRP conjugate into all the wells.
5. Incubate for 2 hours at room temperature.
6. Aspirate the liquid from each well.
7. Wash the plate 3 times by:
 - Dispensing 0.4 ml of working wash solution into each well
 - Aspirating the contents of each well
8. Pipette 100 µl of the chromogen solution into each well within 15 minutes of the washing step.
9. Incubate the microplate for 30 minutes at room temperature and avoid direct sunlight.
10. Pipette 100 µl of stop solution into each well.
11. Read the absorbences at 450 nm (reference filter 630 nm or 650 nm) within 1 hour and calculate the results as described in section X.

X. CALCULATION OF RESULTS

1. Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
2. Calculate the mean of the duplicate determinations.
3. On semi-logarithmic or linear graph paper plot the OD values (ordinate) for each calibrator against the corresponding concentration of OST (abscissa) and draw a calibration curve through the calibrator points by connecting the plotted points with straight lines.
4. Read the concentration for each control and sample by interpolation on the calibration curve.
5. Computer assisted data reduction will simplify these calculations. If automatic result processing is used, a 4 parameter logistic function curve fit is recommended.

If Trasylol is added to the samples (100 µl/ml), sample values have to be multiplied by 1.1.

XI. TYPICAL DATA

The following data are for illustration only and should never be used instead of a real time calibration curve.

OST ELISA		OD
Calibrator	0.0 ng/ml	0.033
	1.56 ng/ml	0.118
	4.1 ng/ml	0.229
	12.7 ng/ml	0.641
	31.5 ng/ml	1.420
	75 ng/ml	2.415

XII. PERFORMANCE AND LIMITATIONS

A. Detection Limit

Twenty zero calibrators were assayed along with a set of other calibrators. The detection limit, defined as the apparent concentration two standard deviations above the average OD at zero binding, was 0.08 ng/ml.

B. Specificity

This method detects intact human osteocalcin. N-terminal and C-terminal fragments, tested at the maximum levels found in normal and pathological samples, were added to a low calibrator and a high value calibrator. No cross-reactivity was observed at these concentrations.

C. Precision

INTRA-ASSAY				INTER-ASSAY			
Serum	N	<X> +/- SD (ng/ml)	CV (%)	Serum	N	<X> +/- SD (ng/ml)	CV (%)
A	20	11.4 +/- 0.5	4.7	A	20	11.8 +/- 0.4	3.5
B	20	28.2 +/- 0.28	3.1	B	20	27.7 +/- 1.55	5.6

SD: Standard Deviation; CV: Coefficient of Variation

D. Accuracy

RECOVERY TEST

Sample	Added OST (ng/ml)	Recovered OST (ng/ml)	(%) Recovery
Serum	1.4	1.55	111
	4.04	4	99
	8.4	8.3	99
	15	14.5	97
	31	31	100
	64.6	64.4	99

DILUTION TEST

Sample	Dilution	Theoretical Concentration (ng/ml)	Measured Concentration (ng/ml)
1	1/1	-	28.6
	1/2	14.3	14.2
	1/4	7.1	7.1
	1/8	3.6	3.4
	1/16	1.8	1.4
2	1/1	-	30.8
	1/2	15.4	15
	1/4	7.7	7.7
	1/8	3.8	3.7

Samples were diluted with zero calibrator.

E. Hook effect

A sample spiked with OST up to 625 ng/ml gives higher OD's than the last calibrator point.

XIII. INTERNAL QUALITY CONTROL

- If the results obtained for control 1 and/or control 2 are not within the range specified on the vial label, the results cannot be used unless a satisfactory explanation for the discrepancy has been given.
- If desirable, each laboratory can make its own pools of control samples, which should be kept frozen in aliquots. Controls which contain azide will interfere with the enzymatic reaction and cannot be used.
- Acceptance criteria for the difference between the duplicate results of the samples should rely on Good Laboratory Practices.
- It is recommended that controls be routinely assayed as unknown samples to measure assay variability. The performance of the assay should be monitored with quality control charts of the controls.
- It is good practice to visually check the curve fit selected by the computer.

XIV. REFERENCE INTERVALS

These values are given only for guidance; each laboratory should establish its own normal range of values. Normal values are expected between 5 to 25 ng/ml.

XV. PRECAUTIONS AND WARNINGS

Safety

For research use only. This kit is not for use in diagnostic procedures.

The human blood components included in this kit have been tested by European approved and/or FDA approved methods and found negative for HBsAg, anti-HCV, and anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS, or other infections. Therefore, handling of reagents and serum samples should be in accordance with local safety procedures.

All animal products and derivatives have been collected from healthy animals. Bovine components originate from countries where BSE has not been reported. Nevertheless, components containing animal substances should be treated as potentially infectious.

Avoid any skin contact with the reagents; stop solution contains HCl and the chromogen solution contains TMB and H₂O₂. In cases of contact, wash thoroughly with water.

Do not smoke, drink, eat, or apply cosmetics in the working area. Do not pipette by mouth. Use protective clothing and disposable gloves.

XVI. BIBLIOGRAPHY

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XVII. SUMMARY OF PROTOCOL

	CALIBRATORS (μl)	SAMPLE(S) CONTROLS (μl)
Calibrators (0-5)	25	-
Samples, Controls	-	25
Working HRP conjugate	100	100
Incubate for 2 hours at RT. Aspirate the contents of each well. Wash 3 times with 0.4 ml of working wash solution. Aspirate the contents of each well.		
Chromogen solution (within 15 min of washing steps)	100	100
Incubate for 30 min at RT – avoid direct sunlight.		
Stop solution	100	100
Read on a microplate reader and record the absorbance of each well at 450 nm (versus 630 or 650 nm) within 1 hr of adding stop solution.		