



Osteocalcin (1-43/49) ELISA

For the quantitative determination of human osteocalcin (1-43) (N-terminal or mid-regional) and human osteocalcin (1-49) (intact) in serum or plasma

Please read carefully due to Critical Changes, e.g., Wash concentrate volume and dilution.

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

Catalog Number:	43-OSNHU-E01
Size:	96 wells
Version:	1/ US/ JAN2009 - ALPCO 2/1/2010

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INTENDED USE

This ELISA (enzyme-linked immunosorbent assay) kit is intended for the quantitative determination of both human osteocalcin (1-49) [intact] and human osteocalcin (1-43) [N-terminal or mid-regional] levels in samples. This kit is useful for assessing the bone formation activity or osteoblast activity associated with changes in the rate of bone turnover in metabolic bone diseases, such as osteoporosis, primary hyperparathyroidism, hyperthyroidism, Paget's disease, and renal osteodystrophy. This kit is for research use only. It is not for use in diagnostic procedures.

INTRODUCTION

Osteocalcin [also known as bone Gla protein (BGP)] is a major noncollagenous protein found in bone and dentin. The synthesis of osteocalcin involves vitamin K and vitamin D₃. Freshly synthesized osteocalcin is partly released into the blood stream and partly incorporated into the bone matrix. Both osteocalcin (1-49) and its fragments, including osteocalcin (1-43), are released into the blood stream. Serum osteocalcin (1-43) is also generated by catabolic breakdown of osteocalcin (1-49) in the circulation, liver, and kidney, as well as by degradation during storage of samples; a labile six-amino acid C-terminal sequence is easily cleaved off at room temperature. There are several studies that have confirmed the combined measurement of intact and the much more stable N-terminal/mid-regional osteocalcin [osteocalcin (1-43/49)] is useful for research, as the combined measurement may contribute to a more accurate assessment of the bone turnover rate.

As osteocalcin is manufactured by osteoblasts, it is often used as a biochemical marker, or biomarker, for the bone formation process. It has been routinely observed that higher serum osteocalcin levels are relatively well correlated with increases in bone mineral density (BMD) during treatment with anabolic bone formation drugs for osteoporosis, such as Forteo. In many studies, osteocalcin is used as a preliminary biomarker on the effectiveness of a given drug on bone formation.

ASSAY PRINCIPLE

This ELISA is designed, developed, and produced for the quantitative measurement of human osteocalcin (1-49) and (1-43) in serum or plasma samples. The assay utilizes the two-site "sandwich" technique with two selected antibodies that bind to different epitopes of human osteocalcin.

Assay standards, controls, and samples are added directly to the wells of a microtiter plate that is coated with streptavidin. Subsequently, a mixture of biotinylated human osteocalcin N-terminal region specific polyclonal antibody and a peroxidase labeled human osteocalcin 20 - 43 region specific monoclonal antibody is added to each well. After the first incubation period, a "sandwich" of "biotinylated antibody - human osteocalcin - HRP - monoclonal antibody" is formed and this immunocomplex is captured on the wall of the microtiter plate via streptavidin - biotin affinity binding. The unbound monoclonal antibodies and buffer matrix are removed in the subsequent washing step. A substrate solution in a timed reaction is then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to the wall of each microtiter well is directly proportional to the amount of human osteocalcin in the test sample. A standard curve is generated by plotting the absorbance versus the respective human osteocalcin concentration for each standard on a point-to-point or 4 parameter curve fit. The concentration of human osteocalcin in the test samples is determined directly from this standard curve.

REAGENTS: Preparation and Storage

This test kit must be stored at 2 – 8°C upon receipt. For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

Prior to use allow all reagents to come to room temperature. Regents from different kit lot numbers should not be combined or interchanged.

1. Streptavidin Coated Microplate (Part No. 10040)

One microplate with 12 x 8 strips (96 wells total) coated with streptavidin. The plate is framed and sealed in a foil zipper bag with a desiccant. This reagent should be stored at 2-8°C and is stable until the expiration date on the kit box.

2. HRP Conjugated Osteocalcin Antibody (Part No. 30288)

One vial contains 1.2 mL of HRP conjugated monoclonal anti-human osteocalcin (20-43) antibody in a stabilized protein matrix. This reagent must be diluted with biotinylated antibody before use. This reagent should be stored at 2-8°C and is stable until the expiration date on the kit box.

3. Biotinylated Osteocalcin Antibody (Part No. 30289)

Two bottles, each containing 12 mL of biotinylated anti-human osteocalcin N-terminal region specific antibody in a stabilized protein matrix. This reagent is ready to be used for dilution of the HRP conjugated osteocalcin antibody. This reagent should be stored at 2-8°C and is stable until the expiration date on the kit box.

4. Wash Concentrate (Part No. 10010)

One bottle contains 30 mL of a 30 fold concentrate. Before use the contents must be diluted with 870 mL of distilled water and mixed well. Upon dilution this yields a working wash solution containing a surfactant in phosphate buffered saline with a nonazide, non-mercury preservative. The diluted wash solution may be stored at room temperature and is stable until the expiration date on the kit box.

5. HRP Substrate (Part No. 10020)

One bottle contains 22 mL of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2-8°C and is stable until the expiration date on the kit box.

6. Stop Solution (Part No. 30357)

One bottle contains 12 mL of sulfuric acid. This reagent may be stored at 2-8°C or room temperature and is stable until the expiration date on the kit box.

7. Standards (Part No. 30291 – 30296)

Six vials, each containing human osteocalcin in a lyophilized bovine serum based matrix with a non-azide, non-mercury preservative. **Refer to the vial labels for the exact concentration of each standard.** These reagents should be stored at 2-8°C and are stable until the expiration date on the kit box.

8. Controls (Part No. 30297 – 30298)

Two vials, each containing human osteocalcin in a lyophilized bovine serum based matrix with a non-azide, non-mercury preservative. Refer to the vial labels for the exact concentration range of each control. Both controls should be stored at 2-8°C and are stable until the expiration date on the kit box.

SAFETY PRECAUTIONS

The reagents must be used in a professional laboratory. Source material for reagents containing bovine serum was derived in the contiguous 48 United States. It was only obtained from healthy donor animals maintained under veterinary supervision and found free of contagious diseases. Wear gloves while performing this assay and handle these reagents as if they were potentially infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Sulfuric acid may cause severe irritation on contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Serum or plasma sample collection tubes.
2. Precision single channel pipettes capable of delivering 25; 100; 200; and 1,000 µL.
3. Repeating dispenser suitable for delivering 100 and 200 µL.
4. Disposable pipette tips suitable for dispensing the above volumes.
5. Disposable 12 x 75 mm or 13 x 100 mm plastic test tubes.
6. Disposable plastic 1,000 mL bottle with cap.
7. Aluminum foil.
8. Deionized or distilled water.
9. Plastic microtiter well cover or polyethylene film.
10. ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
11. Spectrophotometric microplate reader capable of reading absorbance at 450 nm.
12. ELISA plate shaker.

SAMPLE COLLECTION

Only 50 µL of human serum or plasma is required for the measurement of human osteocalcin in duplicate. No special preparation of the individual is necessary prior to sample collection. Whole blood should be collected and it must be allowed to clot for a minimum of 30 minutes at room temperature before the serum is separated by centrifugation (850 – 1,500 x g for 10 minutes). The serum should be separated from the clot within three hours of blood collection and transferred to a clean test tube. Serum samples can be stored at 2-8°C or room temperature for 6 days until measurement. For longer storage samples should be stored frozen (-20°C). Do not exceed 3 freeze-thaw cycles. It is necessary to avoid hemolysis while conducting the sample collection procedure.

ASSAY PROCEDURE

1. Reagent Preparation

- (1) Prior to use, allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
- (2) Wash Concentrate must be diluted to working solution prior to use. Please see REAGENTS section for details.
- (3) Reconstitute all assay standards and controls by adding **0.5 mL** of distilled or demineralized water to each vial. Allow the standards and controls to sit undisturbed for 5 minutes, and then mix well by inversion or gentle vortexing. One must make sure that all solids are dissolved completely prior to use. These reconstituted standards and controls must be stored at -18°C or below. Do not exceed 3 freeze-thaw cycles.

2. Assay Procedure

- (1) Place a sufficient number of streptavidin coated microwell strips in a holder to run the standards, controls, and unknown samples in duplicate. The unused strips should be resealed in the bag with a desiccant and stored at 2-8°C.
- (2) Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3
A	STD 1	STD 5	SAMPLE 1
B	STD 1	STD 5	SAMPLE 1
C	STD 2	STD 6	SAMPLE 2
D	STD 2	STD 6	SAMPLE 2
E	STD 3	C 1	SAMPLE 3
F	STD 3	C 1	SAMPLE 3
G	STD 4	C 2	
H	STD 4	C 2	

- (3) Prepare working antibody *mixture* by 1:21 fold dilution of the HRP Conjugated Osteocalcin Antibody (Part No. 30288) with the Biotinylated Osteocalcin Antibody (Part No. 30289). The following is a table that outlines the relationship between the strips used and the amount of antibody *mixture* prepared.

Strip Qty	30288 Solution	30289 Solution
1	100 μ L	2 mL
2	200 μ L	4 mL
3	300 μ L	6 mL
4	400 μ L	8 mL
5	500 μ L	10 mL
6	600 μ L	12 mL
7	700 μ L	14 mL
8	800 μ L	16 mL
9	900 μ L	18 mL
10	1000 μ L	20 mL
11	1100 μ L	22 mL
12	1200 μ L	24 mL

Note: This antibody *mixture* should be prepared immediately before running the assay.

- (4) Add **25 μ L** of standards, controls, and serum/plasma samples to the designated microwells.
- (5) Add **200 μ L** of the working antibody *mixture* (see step 3) to each well.
- (6) Cover the plate with a plate sealer and aluminum foil to avoid exposure to light.
- (7) Incubate the plate at room temperature, shaking 350 rpm +/- 100 rpm for **1 hour**.
- (8) Remove the aluminum foil and plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing 350 μ L - 400 μ L of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
- (9) Add **200 μ L** of HRP substrate to each of the wells.
- (10) Cover the plate with a new plate sealer and aluminum foil to avoid exposure to light.
- (11) Incubate the plate at room temperature, static for **20 minutes**. (*This incubation period may be reduced to 8-15 minutes if a lower OD reading is demanded to fit to the plate reader's specifications.*)
- (12) Remove the aluminum foil and plate sealer. Add **50 μ L** of Stop Solution to each of the wells. Mix gently.
- (13) Read the absorbance at **450 nm** within 10 minutes in a microplate reader.

NOTE: *In cases where extremely low background is required, one can set the instrument to dual wavelength measurement at 450 nm with background wavelength correction set at 595 nm, 620 nm, or 630 nm.*

PROCEDURAL NOTES

1. It is recommended that all standards, controls, and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results.
2. Keep light sensitive reagents in the original amber bottles.
3. Store any unused streptavidin coated strips in the foil zipper bag with desiccant to protect from moisture.
4. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
5. Observing incubation times or temperatures other than those stated in this insert may affect the results.
6. Avoid air bubbles in the microwells as this could result in lower binding efficiencies and higher CV% of duplicate readings.
7. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.
8. Prepare a calibration curve for each run; do not use data from previous runs.
9. To avoid cross-contamination, use a clean disposable pipette tip for the addition of each reagent and sample.

INTERPRETATION OF RESULTS

1. Calculate the average absorbance for each pair of duplicate test results.
2. Subtract the average absorbance of the zero standard from the average absorbance of all other readings to obtain the corrected absorbance.
3. The standard curve is generated by the corrected absorbance of all standard levels on the ordinate against the standard concentration on the abscissa using point-to-point or log-log paper. Appropriate computer assisted data reduction programs (e.g., Point-to-Point, 4-Parameter) may also be used for the calculation of results.

The human osteocalcin concentrations for the controls and the samples are read directly from the standard curve using their respective corrected absorbances. If log-log graph paper or a computer assisted data reduction program utilizing logarithmic transformation is used, samples having corrected absorbances between the second standard and the next highest standard should be calculated by the following formula:

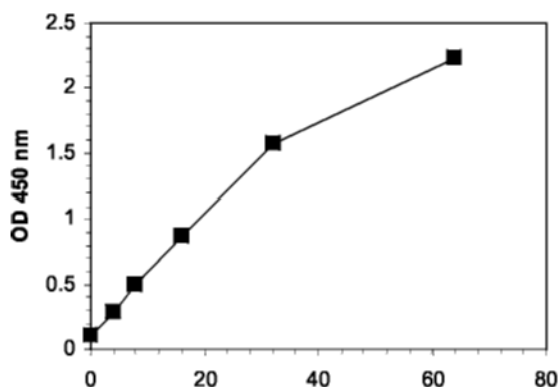
$$\text{Sample Value} = \frac{\text{Corrected absorbance (Unknown sample)}}{\text{Corrected absorbance (2}^{\text{nd}} \text{ STD)}} \times \text{Value of the 2}^{\text{nd}} \text{ STD}$$

EXAMPLE DATA AND STANDARD CURVE

Typical absorbance data and the resulting standard curve from this human osteocalcin ELISA are represented. **This curve should not be used in lieu of a standard curve run with each assay.**

Sample	Results		
	Average	Corrected	
0 ng/mL	0.112	0.000	
4 ng/mL	0.279	0.167	
8 ng/mL	0.494	0.382	
16 ng/mL	0.866	0.754	
32 ng/mL	1.570	1.458	
64 ng/mL	2.232	2.120	
Control 1	0.363	0.251	5.26 ng/mL
Control 2	0.663	0.551	11.69 ng/mL

Human Osteocalcin (1-43/49) ELISA



Human Osteocalcin Standards (ng/mL)

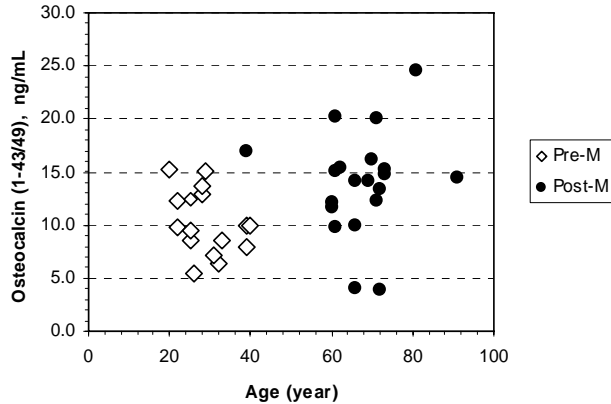
EXPECTED VALUES

Forty serum samples from normal healthy adults, ages 26 – 58, were collected and measured with this ELISA. The normal osteocalcin range was found to be 3.8 – 25.3 ng/mL, the mean osteocalcin level of this group was 11.7 ng/mL (median: 11.4 ng/mL), and the Standard Deviation was 3.8 ng/mL. The ninety-fifth percentile normal high cut-off is 17 ng/mL based on this study group.

A validation study of pre- and post-menopausal woman, as well as a group of male subjects, indicated a significant differentiation of serum osteocalcin levels in post-menopausal woman as compared to the other two groups with this ELISA. The data is summarized in the following table and figure.

	Premenopausal Woman (n = 16)	Postmenopausal Woman (n = 19)	Male (n = 15)
Age			
Mean	29.0	68.7	50.3
SD	6.3	7.9	9.9
Range	21 – 40	60 – 91	37 – 76
Osteocalcin (1-43/49) ng/mL			
Mean	10.3	13.8	10.8
SD	3.0	5.0	3.6
Range	5.4 – 15.2	3.9 – 21.6	5.4 – 15.1

Pre- and Post-menopausal Female



Forty serum samples from subjects with end stage renal diseases on hemodialysis were also measured with this ELISA. Except for one subject, all other 39 subjects showed osteocalcin values above the normal high cut-off ranging from 21 ng/mL to 119 ng/mL with a mean value of 60.6 ng/mL (median: 59.6 ng/mL, SD: 26.2 ng/mL).

LIMITATIONS OF THE PROCEDURE

1. An abnormally high osteocalcin value is likely to indicate a more significant bone turnover condition in a subject. When a sample value reads higher than the highest standard, it is recommended to dilute the sample and assay again.
2. Different age groups and genders may show different normal ranges of osteocalcin.
3. Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.

QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls.

PERFORMANCE CHARACTERISTICS

Sensitivity

The sensitivity of this human osteocalcin ELISA, as determined by the 95% confidence limit on 8 replicate determinations of both zero and level 2 standards, is approximately 0.31 ng/mL.

High Dose "hook" effect

This assay showed it did not have any high dose "hook" for sample osteocalcin levels up to 1,250 ng/mL.

Precision

The intra-assay precision is validated by measuring two samples in a single assay with 16 replicate determinations.

Mean Osteocalcin Value (ng/mL)	CV (%)
11.9	4.7
40.2	5.0

The inter-assay precision is validated by measuring two control samples in duplicate in 6 individual assays.

Mean Osteocalcin Value (ng/mL)	CV (%)
5.6	8.3
11.9	5.7

Linearity

Two serum samples from dialysis subjects were diluted with a BSA based 0.01M phosphate, 0.15M sodium chloride buffer matrix and assayed. The results in ng/mL are as follows:

#	DILUTION	OBSERVED VALUE	EXPECTED VALUE	RECOVERY
1	Neat	69.6	-	-
	1:2	34.5	34.8	99%
	1:4	15.1	17.4	87%
2	Neat	42.1	-	-
	1:2	21.4	21.1	101%
	1:4	10.4	10.5	99%

Recovery

Two serum samples are spiked with three assay standards in equal volume (1 volume + 1 volume mixture) and assayed. The results in ng/mL are as follows:

#	Original Value	Spiked Sample Value	Observed Value	Expected Value	Recovery %
Sample 1					
1	33.4	8	18.5	20.7	89
2	33.4	16	23.8	24.7	96
3	33.4	32	30.4	32.7	93
Sample 2					
4	15.7	8	11.4	11.9	96
5	15.7	16	15.3	15.9	96
6	15.7	32	24.4	23.9	102

REFERENCES

1. Rosenquist C, Qvist P, Bjarnason N, Christiansen C. Measurement of a more stable region of osteocalcin in serum by ELISA with two monoclonal antibodies. *Clin Chem*. 1995 Oct; 41 (10):1439-45.
2. Takahashi M, Kushida K, Nagano A, Inoue T. Comparison of the analytical and clinical performance characteristics of an N-MID versus an intact osteocalcin immunoradiometric assay. *Clin Chim Acta*. 2000 Apr; 294 (1-2):67-76.
3. Nagasue K, Inaba M, Okuno S, Kitatani K, Imanishi Y, Ishimura E, Miki T, Kim M, Nishizawa Y. Serum N-terminal midfragment vs. intact osteocalcin immunoradiometric assay as markers for bone turnover and bone loss in hemodialysis patients. *Biomed Pharmacother*. 2003 Mar; 57 (2):98-104.
4. Garnero P, Grimaux M, Seguin P, Delmas PD. Characterization of immunoreactive forms of human osteocalcin generated *in vivo* and *in vitro*. *J Bone Miner Res*. 1994 Feb; 9 (2):255-64.

Short Assay Procedure:

1. Add 25 μ L of standards, controls, and samples to the designated wells.
 2. Add 200 μ L of antibody mixture to each of the wells.
 3. Cover with plate sealer and aluminum foil and incubate for 1 hr at RT, shaking 350 rpm.
 4. Wash each well 5 times with 350 - 400 μ L of working wash solution.
 5. Add 200 μ L of HRP Substrate to each of the wells.
 6. Cover with plate sealer and aluminum foil and incubate 20 min at RT, static.
 7. Add 50 μ L of Stop Solution to each of the wells.
 8. Read the absorbance at 450 nm within 10 min.
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