Activated Carboxylated Osteocalcin ELISA

Catalog Number:
OST31-K01 (1 x 96 wells)
For Research Use Only. Not for use in diagnostic procedures.

v. 2.0 (07.19.22)

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I. Intended Use

This ELISA (enzyme-linked immunosorbent assay) kit is intended for the quantitative determination of both human carboxylated osteocalcin (1-49) and carboxylated osteocalcin (1-43) (also referred as N-terminal & mid-regional osteocalcin) levels in test samples. This kit is for research use only.

II. Introduction

Osteocalcin [also 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 bloodstream 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 in vitro degradation during storage of samples, because of a labile six-amino acid C-terminal sequence that, in vitro at room temperature, is easily cleaved off. There are several studies that have confirmed the measurement of the much more stable N-terminal and mid-regional osteocalcin [osteocalcin (1-43/49)] as being clinically useful, which 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.

III. Assay Principle

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

Assay calibrators, controls and patient samples are added directly to 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 also captured to the wall of microtiter plate via a 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 a test sample. A calibration curve is generated by plotting the absorbance versus the respective human osteocalcin concentration for each calibrator on point-to-point or 4 parameter curve fit. The concentration of human osteocalcin in test samples is determined directly from this calibration curve.

IV. Reagents: Preparation and Storage

The Eagle Biosciences Activated Carboxylated Osteocalcin **ELISA Assay 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.

1. Streptavidin Coated Microplate

Microplate coated with streptavidin.

Qty: 1 x 96 well microplate
Storage: 2 – 8°C
Preparation: Ready to Use

2. HRP-Conjugated Osteocalcin Antibody

HRP-conjugated monoclonal anti-human osteocalcin (20-43) antibody in a stabilized protein matrix.

Qty: 1 x 1.2 mL
Storage: 2 – 8°C
Preparation: 21X Concentrate. This reagent must be diluted with Biotinylated Osteocalcin Antibody before use.

3. Biotinylated Osteocalcin Antibody

Biotinylated anti-human osteocalcin N-terminal region specific antibody in a stabilized protein matrix.

Qty: 2 x 12 mL
Storage: 2 – 8°C
Preparation: Ready to Use

4. ELISA Wash Concentrate

Surfactant in a phosphate buffered saline with non-azide preservative.

Qty: 1 x 30 mL
Storage: 2 – 25°C
Preparation: 30X Concentrate. The contents must be diluted with 870 mL distilled water and mixed well before use.

5. ELISA HRP Substrate

Tetramethylbenzidine (TMB) with stabilized hydrogen peroxide.

Qty: 1 x 22 mL
Storage: 2 – 8°C
Preparation: Ready to Use

6. ELISA Stop Solution

1.0 M sulfuric acid
Qty: 1 x 12 mL
Storage: 2 – 25°C
Preparation: Ready to Use

7. Human Osteocalcin Calibrators Levels 1 to 6
Human osteocalcin in a lyophilized bovine serum-based matrix with a non-azide, non-mercury preservative. Refer to each vial for exact concentration.

Qty: 6 x Vials
Storage: 2 – 8°C (Lyophilized), <-20°C(Reconstituted) Do not exceed 3 freeze-thaw cycles.
Preparation: Must be reconstituted with 0.5 mL of demineralized water, allowed to sit for 10 minutes, and then mixed by inversions or gentle vortexing. Make sure that all solids are dissolved completely prior to use.

8. Human Osteocalcin Controls
Human osteocalcin in a lyophilized bovine serum-based matrix with a non-azide, non-mercury preservative. Refer to each vial for exact concentration.

Qty: 2 x Vials
Storage: 2 – 8°C (Lyophilized), <-20°C(Reconstituted) Do not exceed 3 freeze-thaw cycles.
Preparation: Must be reconstituted with 0.5 mL of demineralized water, allowed to sit for 10 minutes, and then mixed by inversions or gentle vortexing. Make sure that all solids are dissolved completely prior to use.

V. Safety Precautions

The reagents are for research only. Source material which contains reagents of bovine serum albumin was derived in the contiguous 48 United States. It was obtained only 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 hydrogen peroxide, or sulfuric acid. 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.

VI. Materials Required but not Provided

1. Serum or plasma sample collection tube.
2. Precision single channel pipettes capable of delivering 25 µL, 100 µL, 200 µL, and 1000 µL etc.
3. Repeating dispenser suitable for delivering 100 µL and 200 µL.
4. Disposable pipette tips suitable for above volume dispensing.
5. Disposable 12 x 75 mm or 13 x 100 plastic test tubes.
6. Disposable plastic 1000 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.

VII. Specimen Collection

Only 50 µL of human serum or plasma sample is required for human osteocalcin measurement in duplicate. Samples should not be taken from patients taking biotin-containing multivitamins or dietary supplements at least 48 hours prior to specimen collection. Whole blood should be collected and must be allowed to clot for minimum 30 minutes at room temperature before the serum is separated by centrifugation (850 – 1500xg 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 sample is allowed to be stored at 2-8°C or room temperature for 6 days until measurement. Sample should be stored in frozen condition (< -20°C) for longer storage. Avoid more than three freeze-thaw cycles of specimen. It is necessary to take care in the sample collection procedure to avoid haemolysis.

VIII. Assay Procedure

1. Reagent Preparation

1. Prior to use allow all reagents to come to room temperature (20-25 ºC). Reagents from different kit lot numbers should not be combined or interchanged.
2. ELISA Wash Concentrate must be diluted to working solution prior use. Please see REAGENTS section for details.
3. Reconstitute all assay calibrators and controls by adding 0.5 mL of deminerialized water to each vial. Allow the calibrators and controls to sit undisturbed for 10 minutes, and then mix well by inversions or gentle vortexing. Make sure that all solids are dissolved completely prior to use. These reconstituted calibrators and controls must be stored at -20°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 calibrators and controls, and samples in duplicate.
2. Test Configuration

Row	Strip 1	Strip 2	Strip 3
A	Calibrator Level 1	Calibrator Level 5	SAMPLE 1
B	Calibrator Level 1	Calibrator Level 5	SAMPLE 1
C	Calibrator Level 2	Calibrator Level 6	SAMPLE 2
D	Calibrator Level 2	Calibrator Level 6	SAMPLE 2
E	Calibrator Level 3	Control 1	SAMPLE 3
F	Calibrator Level 3	Control 1	SAMPLE 3
G	Calibrator Level 4	Control 2	SAMPLE 4
H	Calibrator Level 4	Control 2	SAMPLE 4

3. Prepare the antibody working solution by 1:21 fold dilution of the HRP antibody with the Biotinylated Antibody. For each strip, it is required to mix 2 mL of the biotinylated antibody with 100 µL of the HRP antibody in a clean test tube. *Note: This antibody working solution should be freshly prepared.*
4. Add **25 µL** of calibrators and controls, and samples into the designated microwells.
5. Add **200 µL** of antibody working solution into each microwell.
6. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25 ºC)** with **shaking at 350 to 450 rpm** for **60 minutes.**
7. Remove the plate sealer. Aspirate the contents of each well. Wash each well **5 times** by dispensing **350 µL** of diluted wash solution into each well, and then completely aspirate the contents. Alternatively, an automated microplate washer can be used.
8. Add **200 µL** of ELISA HRP Substrate into each of the wells. Mix by gently tapping the plate.
9. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25 ºC)** for **20 minutes.**
10. Remove the aluminum foil and plate sealer. Add **50 µL** of ELISA Stop Solution into each of the wells. Mix by gently tapping the plate.
11. Read the absorbance at **450 nm** within **10 minutes** with a microplate reader.

IX. **Procedure** Notes

1. It is recommended that all calibrators, 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. Incubation times or temperatures other than those stated in this insert may affect the results.
6. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
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.

X. Interpretation of Results

1. Calculate the average absorbance for each pair of duplicate test results.
2. Subtract the average absorbance of the zero calibrator from the average absorbance of all other readings to obtain corrected absorbance.
3. The calibration curve is generated by the corrected absorbance of all calibrator levels on the ordinate against the calibrator 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.
4. The sample human osteocalcin concentrations for the controls and the patient samples are read directly from the calibration curve using their respective corrected absorbance.

XI. Limitation of the Procedure

1. An abnormally high osteocalcin value is likely to indicate a more significant bone turnover condition of a patient. For sample values reading greater than the highest calibrator, it is recommend to re-assay sample with dilution.
2. Different age group and gender may show a different normal range of osteocalcin.
3. Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.

XII. Quality Control

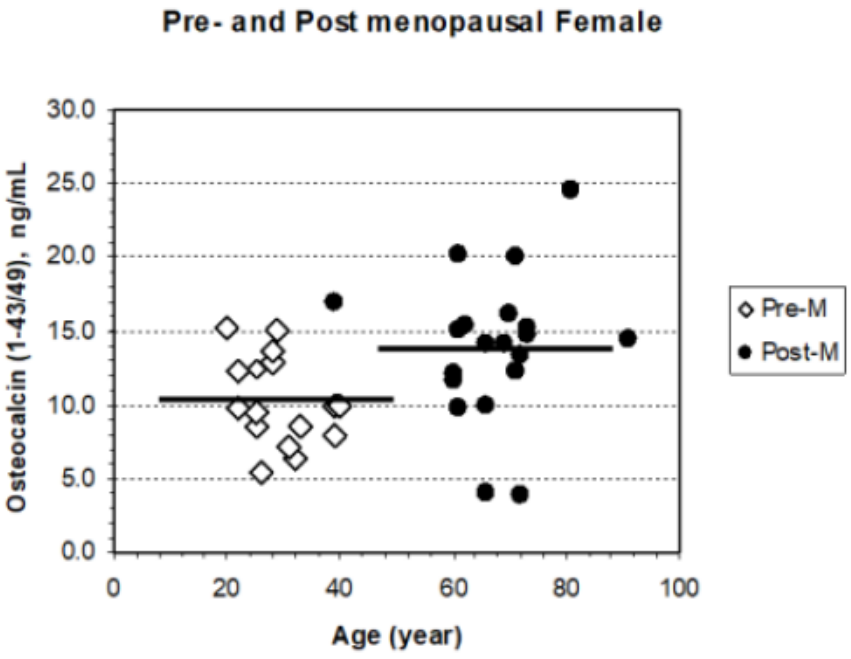
To assure the validity of the results each assay should include adequate controls.

XIII. Expected Values

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

A validation study of pre- and post-menopausal women, as well as a group of male subjects, indicated a well-differentiated serum osteocalcin level of post-menopausal women from other two groups with this ELISA. The data is summarized in the following table and figure.

	Premenopausal Women (n = 16)	**Postmenopausal Women (n = 19)**	**Male (n = 15)**
Mean	29.0	68.7	50.3
Standard Deviation	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
Standard Deviation	3.0	5.0	3.6
Range	5.4 – 15.2	3.9 – 21.6	5.4 – 15.1



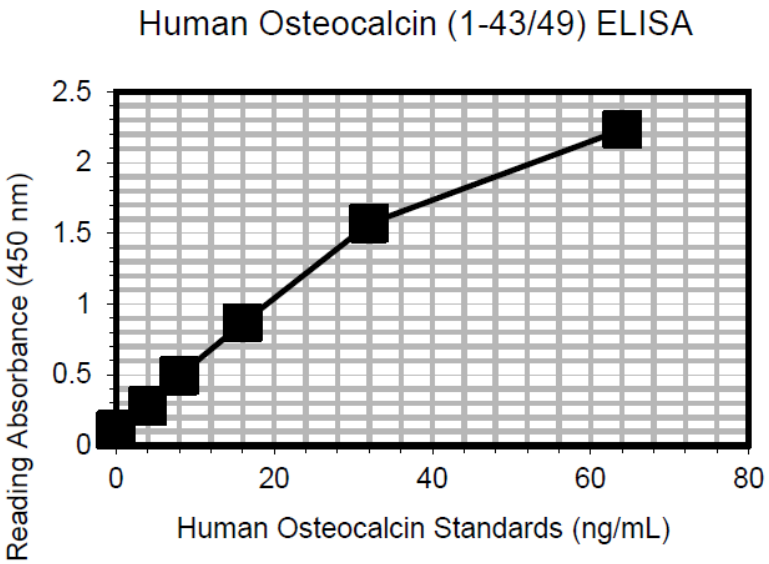
Forty serum samples from patients with end-stage renal diseases on hemodialysis were also measured with this ELISA. Except for one patient, all other 39 patients showed their osteocalcin values above the normal high cut-off ranging from 21 ng/mL to 119 ng/mL with a mean value of 60.6 ng/mL (median: 59.6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

XIV. Example Data

A typical absorbance data and the resulting calibration curve from human osteocalcin ELISA are represented.

Note: This curve should not be used in lieu of calibration curve run with each assay.

Well ID	Reading Absorbance (450 nm)		Concentration (ng/mL)
	Average	Corrected	
Calibrator Level 1: 0 ng/mL	0.112	0.000	
Calibrator Level 2: 4 ng/mL	0.279	0.167	
Calibrator Level 3: 8 ng/mL	0.494	0.382	
Calibrator Level 4: 16 ng/mL	0.866	0.754	
Calibrator Level 5: 32 ng/mL	1.570	1.458	
Calibrator Level 6: 64 ng/mL	2.232	2.120	
Control 1	0.363	0.251	5.26
Control 2	0.663	0.551	11.69



XV. 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 calibrators is approximately 0.31 ng/mL.

Hook Effect

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

Specificity

This assay shows less than 15% cross reactivity to uncarboxylated osteocalcin.

Reproducibility and Precision

The intra-assay precision is validated by measuring two patient samples in a single assay with 16 replicate determinations. The inter-assay precision is validated by measuring two control samples in duplicate in 6 individual assays. The results are as follows:

	Intra-Assay		Inter-Assay	
Sample	**1**	**2**	**1**	**2**
Mean (ng/mL)	11.9	40.2	5.6	11.9
CV (%)	4.7	5.0	8.3	5.7

Linearity

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

Samples	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Sample A	69.6	-	-
50%	34.5	34.8	99
25%	15.1	17.4	87
Sample B	42.1	-	-
50%	21.4	21.1	101
25%	10.4	10.5	99

Spike Recovery

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

Samples	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Sample A	33.4	-	-
+ Level 3	18.5	20.7	89
+ Level 4	23.8	24.7	96
+ Level 5	30.4	32.7	93
Sample B	15.7	-	-
+ Level 3	11.4	11.9	96
+ Level 4	15.3	15.9	96
+ Level 5	24.4	23.9	102

Interfering Substances

Lipids 3000 mg/mL	**Test Osteocalcin (ng/mL)**	**Control Osteocalcin (ng/mL)**	**Dcut (ng/mL)**	**Bias (ng/mL)**	**Bias (%) (dobs)**
	42.6	42.6	3.2	0.0	0.0
	13.1	12.1	1.0	1.0	8.2
	8.8	8.2	0.6	0.6	7.3
Hemoglobin 200 mg/mL	**Test Osteocalcin (ng/mL)**	**Control Osteocalcin (ng/mL)**	**Dcut (ng/mL)**	**Bias (ng/mL)**	**Bias (%) (dobs)**
	42.0	41.3	3.1	0.7	1.7
	13.6	13.4	1.0	0.2	1.5
	9.7	9.1	0.7	0.6	6.6
Hemoglobin 66.6 mg/mL	**Test Osteocalcin (ng/mL)**	**Control Osteocalcin (ng/mL)**	**Dcut (ng/mL)**	**Bias (ng/mL)**	**Bias (%) (dobs)**
	42.3	41.3	3.1	1.0	0.2
	13.5	13.4	1.0	0.1	0.7
	9.4	9.1	0.7	0.3	3.3
Bilirubin 20 mg/mL	**Test Osteocalcin (ng/mL)**	**Control Osteocalcin (ng/mL)**	**Dcut (ng/mL)**	**Bias (ng/mL)**	**Bias (%) (dobs)**
	22.2	22.9	1.7	0.7	3.1
	7.4	7.5	0.6	0.1	1.3
	4.7	4.6	0.3	0.1	2.2

XVI. References

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4. 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
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5. 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

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